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(54) Title: THE RICE ACTIN 2 PROMOTER AND INTRON AND METHODS FOR USE THEREOF		
(57) Abstract <p>The current invention provides regulatory regions from the rice actin 2 gene. In particular, the current invention provides the rice actin 2 promoter and actin 2 intron. Compositions comprising these sequences are described, as well as transformation constructs derived therefrom. Further provided are methods for the expression of transgenes in plants comprising the use of these sequences. The methods of the invention include the direct creation of transgenic plants with the rice actin 2 intron and/or promoter directly by genetic transformation, as well as by plant breeding methods. The actin 2 sequences of the invention represent a valuable new tool for the creation of transgenic plants, preferably having one or more added beneficial characteristics.</p>		

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DESCRIPTION

THE RICE ACTIN 2 PROMOTER AND INTRON AND METHODS FOR USE THEREOF

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

The present invention relates generally to transgenic plants. More specifically, it relates to methods and compositions for transgene expression using regulatory elements from the rice actin 2 gene.

10 2. Description of the Related Art

An important aspect in the production of genetically engineered crops is obtaining sufficient levels of transgene expression in the appropriate plant tissues. In this respect, the selection of promoters for directing expression of a given transgene is crucial. Promoters which are useful for plant transgene expression include those that are inducible, viral, synthetic,
15 constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989).

A number of plant promoters have been described with various expression characteristics. Examples of some constitutive promoters which have been described include the rice actin 1 (Wang *et al.*, 1992), CaMV 35S (Odell *et al.*, 1985), CaMV 19S (Lawton *et al.*, 1987), and *nos*
20 (Ebert *et al.*, 1987).

Examples of tissue specific promoters which have been described include the lectin (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989; Dennis *et al.*, 1984), corn light harvesting complex (Simpson, 1986; Bansal *et al.*, 1992), corn heat shock protein (Odell *et al.*, 1985; Rochester *et al.*, 1986), pea small subunit RuBP
25 carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), truncated

CaMV 35s (Odell *et al.*, 1985), potato patatin (Wenzler *et al.*, 1989), root cell (Conkling *et al.*, 1990), maize zein (Reina *et al.*, 1990; Kriz *et al.*, 1987; Wandelt and Feix, 1989; Langridge and Feix, 1983; Reina *et al.*, 1990), globulin-1 (Belanger and Kriz, 1991), Adh (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990), α -tubulin, cab (Sullivan *et al.*, 1989), PEPCase
5 (Hudspeth & Grula, 1989), R gene complex-associated promoters (Chandler *et al.*, 1989) and chalcone synthase promoters (Franken *et al.*, 1991).

Examples of inducible promoters which have been described include ABA- and turgor-inducible promoters, the promoter of the auxin-binding protein gene (Scwob *et al.*, 1993), the UDP glucose flavonoid glycosyl-transferase gene promoter (Ralston *et al.*, 1988); the MPI
10 proteinase inhibitor promoter (Cordero *et al.*, 1994), and the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler *et al.*, 1995; Quigley *et al.*, 1989; Martinez *et al.*, 1989).

The rice actin 1 promoter constitutes a particularly useful promoter for expression of transgenes in plants (Wang *et al.*, 1992; U.S. Patent No. 5,641,876). The rice actin 1 gene, *Act1*, encode a transcript that is relatively abundant in all rice tissues and at all developmental stages
15 examined. A complete structural analysis of the rice *Act1* gene has led to the identification and localization a 5' intron from the first coding exon of the *Act1* sequence (McElroy *et al.*, 1990a). This 5' intron was found to be essential for the efficient function of the actin 1 promoter.

Plant actin is encoded by a gene family present in all plant species studied to date (Meagher, 1991). In rice, there are at least eight actin-like sequences per haploid genome. Four
20 of the rice actin coding sequences (rice actin 1, 2, 3 and 7) have been isolated and shown to differ from each other in the tissue and stage-specific abundance of their respective transcripts (Reece, 1988; McElroy *et al.*, 1990a; Reece *et al.*, 1990; U.S. Patent No. 5,641,876; Genbank Accession numbers X15865, X15864, X15862, and X15863, respectively). *In situ* histochemical localization of the product of a fusion between the actin 1 promoter and a gus reporter gene in
25 transgenic rice plants revealed that the *Act1* 5' region is active in most, but not all, sporophytic cell types as well as in gametophytic pollen tissues (McElroy *et al.*, 1990b; 1990c). This pattern is believed to reflect an ubiquitous requirement for cytoskeletal components in plant cells (Zhang *et al.*, 1991).

While the above studies have provided an understanding of the coding regions of rice actin genes, little is still known regarding the 5' regulatory regions of all but the rice actin 1 gene. In particular, there has been a failure in the art to identify the structure, sequence and function of the rice actin 2 upstream regulatory regions. The identification and sequencing of these regions could potentially provide valuable new tools for the preparation of transgenic plants.

SUMMARY OF THE INVENTION

Therefore, according to the present invention, there is provided an isolated rice actin 2 promoter. More particularly, there are provided rice actin 2 promoter sequences isolatable from the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2. In specific embodiments, there are provided nucleic acid segments of from about 40 to about 743 contiguous nucleotides, from about 60 to about 743 contiguous nucleotides, from about 125 to about 743 contiguous nucleotides, from about 250 to about 743 contiguous nucleotides, from about 400 to about 743 contiguous nucleotides, or from about 600 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2. Alternatively, the isolated nucleic acid may comprise the entire nucleic acid sequence of SEQ ID NO:2.

In a second embodiment of the present invention, there is provided an isolated rice actin 2 intron. More particularly, there are provided rice actin 2 intron isolatable from the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3. In specific embodiments, there are provided nucleic acid segments of from about 40 to about 1763 contiguous nucleotides, from about 100 to about 1763 contiguous nucleotides, from about 300 to about 1763 contiguous nucleotides, from about 700 to about 1763 contiguous nucleotides, from about 1200 to about 1763 contiguous nucleotides, or from about 1500 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3. Alternatively, the isolated nucleic acid may comprise the entire nucleic acid sequence of SEQ ID NO:3.

Also provided are expression vectors including those where a rice actin 2 promoter is operably linked to a selected gene. It also may be linked to a terminator region. Further, the vector may comprise a genetic element which enhances the expression of said selected gene when said expression vector is stably transformed in the genome of a host plant. The genetic element may be the rice actin 1 intron or the rice actin 2 intron. The expression vector may be a plasmid.

The selected gene may be an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

The selected gene may be a selectable marker gene encoding a protein selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase and anthranilate synthase.

The expression vector may further encode a transit peptide, such as the chlorophyll a/b binding protein transit peptide, the small subunit of ribulose biphosphate carboxylase transit peptide, the EPSPS transit peptide or the dihydrodipicolinic acid synthase transit peptide. The expression vector may encode any of the above-noted selected genes.

Another expression vector of the present invention comprises an isolated rice actin 2 intron. This vector may further comprise a promoter operable in plants and may comprise a terminator. It may comprise a plasmid.

The promoter may be a gamma zein promoter, an oleosin ole16 promoter, a globulin1 promoter, an actin 1 promoter, an actin c1 promoter, a sucrose synthetase promoter, an INOPS promoter, an EMB5 promoter, a globulin2 promoter, a b-32, ADPG-pyrophosphorylase promoter, an Ltp1 promoter, an Ltp2 promoter, an oleosin ole17 promoter, an oleosin ole18 promoter, an actin 2 promoter, a pollen-specific protein promoter, a pollen-specific pectate lyase promoter, an anther-specific protein promoter, an anther-specific gene RTS2 promoter, a pollen-specific gene promoter, a tapetum-specific gene promoter, tapetum-specific gene RAB24 promoter, an anthranilate synthase alpha subunit promoter, an alpha zein promoter, an anthranilate synthase beta subunit promoter, a dihydrodipicolinate synthase promoter, a Thi1 promoter, an alcohol dehydrogenase promoter, a cab binding protein promoter, an H3C4 promoter, a RUBISCO SS starch branching enzyme promoter, an ACCase promoter, an actin3 promoter, an

actin7 promoter, a regulatory protein GF 14-12 promoter, a ribosomal protein L9 promoter, a cellulose biosynthetic enzyme promoter, an S-adenosyl-L-homocysteine hydrolase promoter, a superoxide dismutase promoter, a C-kinase receptor promoter, a phosphoglycerate mutase promoter, a root-specific RCc3 mRNA promoter, a glucose-6 phosphate isomerase promoter, a pyrophosphate-fructose 6-phosphate phosphotransferase promoter, an ubiquitin promoter, a beta-ketoacyl-ACP synthase promoter, a 33kDa photosystem II promoter, an oxygen evolving protein promoter, a 69 kDa vacuolar ATPase subunit promoter, a metallothionein-like protein promoter, a glyceraldehyde-3-phosphate dehydrogenase promoter, an ABA- and ripening-inducible-like protein promoter, a phenylalanine ammonia lyase promoter, an adenosine triphosphatase S-adenosyl-L-homocysteine hydrolase promoter, an α -tubulin promoter, a cab promoter, a PEPCase promoter, an R gene promoter, a lectin promoter, a light harvesting complex promoter, a heat shock protein promoter, a chalcone synthase promoter, a zein promoter, a globulin-1 promoter, an ABA promoter, an auxin-binding protein promoter, a UDP glucose flavonoid glycosyl-transferase gene promoter, an MPI promoter, an actin promoter, an opaque 2 promoter, a b70 promoter, an oleosin promoter, a CaMV 35S promoter, a CaMV 19S promoter, a histone promoter, a turgor-inducible promoter, a pea small subunit RuBP carboxylase promoter, a Ti plasmid mannopine synthase promoter, Ti plasmid nopaline synthase promoter, a petunia chalcone isomerase promoter, a bean glycine rich protein 1 promoter, a CaMV 35S transcript promoter, a Potato patatin promoter, or a S-E9 small subunit RuBP carboxylase promoter.

In another embodiment of the present invention, there is provided a fertile transgenic plant stably transformed with a selected DNA comprising an actin 2 promoter, and optionally a terminator. The fertile transgenic plant may further comprise a selected gene operably linked to the rice actin 2 promoter. Again, the selected gene may be any of the aforementioned genes. The selected DNA may further comprises a genetic element which enhances the expression of said selected gene in said fertile transgenic plant, such as the rice actin 1 intron and rice actin 2 intron. The selected DNA may comprise a transit peptide, including any of those mentioned above. The plant may be a monocotyledonous plant, such as wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane. The plant may be a dicotyledonous plant, such as tobacco, tomato, potato, soybean, sunflower, alfalfa, canola and cotton.

In still another embodiment, there is provided a crossed fertile transgenic plant prepared according to the method comprising the steps of (i) obtaining a fertile transgenic plant comprising a selected DNA comprising an actin 2 promoter; (ii) crossing said fertile transgenic plant with itself or with a second plant lacking said selected DNA to prepare the seed of a crossed fertile transgenic plant comprising said selected DNA; and (iii) planting said seed to obtain a crossed fertile transgenic plant. The plant may be a monocot or a dicot as set out above. In a particular embodiment, the plant is maize.

The crossed fertile transgenic plant may have the selected DNA inherited through a female parent or through a male parent. The second plant may be an inbred plant. The crossed fertile transgenic may be a hybrid. The crossed fertile transgenic may have the actin 2 promoter isolatable from the nucleic acid sequence of SEQ ID NO:2, including those particular segments set forth above. The crossed fertile transgenic plant also may have, as part of the selected DNA, an exogenous gene operably linked to said actin 2 promoter, including any of those set out above. Also included within the present invention are seeds of any of these crossed fertile transgenic plants.

In yet another embodiment, there is provided a crossed fertile transgenic plant prepared according to the method comprising (i) obtaining a fertile transgenic plant comprising a selected DNA comprising an actin 2 intron; (ii) crossing said fertile transgenic plant with itself or with a second plant lacking said selected DNA to prepare seed of a crossed fertile transgenic plant comprising said selected DNA; and (iii) planting said seed to obtain a crossed fertile transgenic plant comprising said selected DNA. The crossed fertile transgenic plant may be monocot or dicot, as set out above, and in particular is maize.

The selected DNA may be inherited through a female parent or through a male parent. The second plant may be an inbred plant and the crossed fertile transgenic plant may be a hybrid. The actin 2 intron may be isolated from the nucleic acid sequence of SEQ ID NO:3. The selected DNA may further comprises an exogenous gene, including any of those set out above. Also included within the invention are seeds of these plants.

In still yet another embodiment, there is provided a method of expressing an exogenous gene in a plant comprising the steps of (i) preparing a construct comprising said exogenous gene

operably linked to an actin 2 promoter; (ii) transforming a recipient plant cell with said construct; and (iii) regenerating a transgenic plant expressing said exogenous gene from said recipient cell. The recipient plant cell may be from a monocot or dicot, particularly maize. The exogenous gene may be any of those set out above.

5 In still yet a further embodiment, there is provided a method of expressing an exogenous gene in a plant comprising the steps of (i) preparing a construct comprising an actin 2 intron and an exogenous gene; (ii) transforming a recipient plant cell with said construct; and (iii) regenerating a transgenic plant expressing said exogenous gene from said recipient cell. The plant may be monocot, dicot, and in particular is maize. The construct may comprise an
10 exogenous gene including those disclosed above, and the promoter includes any of those disclosed above.

In an additional embodiment, there is provided a method of plant breeding comprising the steps of (i) obtaining a transgenic plant comprising a selected DNA comprising an actin 2 promoter; and (ii) crossing said transgenic plant with itself or a second plant. The second plant
15 may be an inbred plant, a monocot, a dicot, and in particular is maize.

In still a further embodiment, there is provided a method of plant breeding comprising the steps of (i) obtaining a transgenic plant comprising a selected DNA comprising an actin 2 intron; and (ii) crossing said transgenic plant with itself or a second plant. The second plant may be an inbred plant, may be monocot or dicot, and in particular is a maize plant.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Sequence of the 5' region of the rice actin 2 gene (SEQ ID NO:1). The actin 2 promoter (SEQ ID NO:2) is indicated by lowercase nucleotides, uppercase nucleotides indicate the actin 2 exon 1, lower case italics indicate the actin 2 intron (SEQ ID NO:3), upper case italics indicate the actin 2 exon 2, and upper case bold italics indicate the actin 2 translation initiation codon.

FIG. 2: Structure of pDGP834. The plasmid pDGP834 (Act2-gus) was constructed for stable transformation in maize. Bp. 34-770 (*Act2* promoter); bp. 771-896 (*Act2* exon 1); bp. 897-2650 (*Act2* intron 1); bp. 2652-2664 (*Act2* exon 2); bp. 2665-4473 (*gus* coding region); bp. 4544-4796 (*nos* terminator).

FIG. 3: Deletion Derivatives of pDGP834. The depicted plasmids are deletion derivatives of pDGP834 that were used in transient assays to delineate the boundaries of expression elements with Act2 promoter/intro activity (only the Act2 promoter, exon 1 and intron 1 are shown below).

FIG. 4: Structure of pSP-Act2.gus.n. Bp. 34-770 (*Act2* promoter); bp. 771-896 (*Act2* exon 1); bp. 897-2650 (*Act2* intron 1); bp. 2652-2664 (*Act2* exon 2); bp. 2665-4473 (*gus* coding region); bp. 4544-4796 (*nos* terminator).

FIG. 5: Structure of pSP-Act2 Δ i.gus.n. Bp. 37-773 (*Act2* promoter); bp. 774-899 (*Act2* exon 1); bp. 900-1779 (*Act2* intron 1); bp. 1781-1793 (*Act2* exon 2); bp. 1794-3602 (*gus* coding region); bp. 3673-3925 (*nos* terminator).

FIG. 6: Structure of pSP-Act2.gus.n2. Bp. 12-529 (*Act2* promoter); bp. 530-655 (*Act2* exon 1); bp. 656-2409 (*Act2* intron 1); bp. 2411-2423 (*Act2* exon 2); bp. 2424-4232 (*gus* coding region); bp. 4303-4555 (*nos* terminator).

FIG. 7: Structure of pSP-Act2.gus.n3. Bp. 28-69 (*Act2* exon 1); bp. 70-2650 (*Act2* intron 1); bp. 2652-2664 (*Act2* exon 2); bp. 2665-4473 (*gus* coding region); bp. 4544-4796 (*nos* terminator).

FIG. 8: Structure of pSP-Act3.gus.n4. Bp. 28-69 (*Act2* exon 1); bp. 70-949 (*Act2* intron 1); bp. 951-963 (*Act2* exon 2); bp. 964-2772 (*gus* coding region); bp. 2834-3095 (*nos* terminator).

FIG. 9: Structure of pSP-Act2.gus.n5. Bp. 47-1477 (*Act2* intron 1); bp. 1479-1491 (*Act2* exon 2); bp. 1492-3300 (*gus* coding region); bp. 3371-3623 (*nos* terminator).

FIG. 10: Structure of pSP-Act2.gus.n6. Bp. 12-1068 (*Act2* intron 1); bp. 1070-1082 (*Act2* exon 2); bp. 1083-2891 (*gus* coding region); bp. 2962-3214 (*nos* terminator).

FIG. 11: Structure of pSP-gus.n. Bp. 41-1849 (*gus* coding region); bp. 1920-2172 (*nos* terminator).

DETAILED DESCRIPTION OF THE INVENTION

The current invention overcomes deficiencies in the prior art by providing novel methods and compositions for the expression of transgenes in plants. In particular, the current invention provides the actin 2 promoter and the actin 2 intron. The actin 2 promoter described herein represents a constitutive promoter which may find wide utility in directing the expression of potentially any gene which one desires to have expressed in a plant. By including the actin 2 intron with transformation constructs comprising an actin 2 promoter, one may increase the efficiency of the expression of genes operably linked to the actin 2 promoter. Alternatively, the actin 2 intron may be included in conjunction with any other plant promoter for the enhancement of the expression of one or more selected genes.

In addition to including the unmodified actin 2 promoter and actin 2 intron sequences, given in SEQ ID NO:2 and SEQ ID NO:3, respectively, the current invention includes derivatives of these sequences. In particular, the instant disclosure provides teaching for one of skill in the art to delimit the functional elements within the actin 2 intron and promoter and to delete any non-essential elements. Functional elements could also be modified to increase the utility of the sequences of the invention for any particular application. For example, a functional region within the actin 2 promoter of the invention could be modified to cause or increase tissue-specific expression.

AS shown in FIG. 3, there are a number of structural elements in the upstream region of the *Act2* gene. Relative to the translation initiation codon at bases 2665-2668, the *Act2* promoter sequence is located at about bases 34 to 770, exon 1 at about bases 771 to 896, intron 1 at about bases 897 to 2650 and exon 2 at about bases 2652 to 2664. The intron 1 5' splice site is located at bases 895-903 and the 3' splice site at bases 2638-2653

I. Plant Transformation Constructs

On important use of the sequences of the invention will be in directing the expression of a transgene which encodes a particular protein or polypeptide product. However, the transgenes also may be non-expressible DNA segments, *e.g.*, transposons such as Ds that do not direct their own transposition, or DNA sequences that interfere with normal transcription or translation, *e.g.*, antisense DNA sequences. The inventors also contemplate that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of the particular selected genes used in accordance with the actin 2 intron and/or actin 2 promoter for transformation of recipient cells will often depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress, or oxidative stress, increased yields, food content and makeup, physical appearance, male sterility, drydown, standability, prolificacy, starch properties or quantity, oil quantity and quality, amino acid or protein composition, and the like.

In certain embodiments, the present inventors contemplate the transformation of a recipient cell with more than transformation construct. Two or more transgenes can be created in a single transformation event using either distinct selected-gene encoding vectors, or using a single vector incorporating two or more gene coding sequences. Of course, any two or more transgenes of any description, such as those conferring, for example, herbicide, insect, disease

(viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties or quantity, oil quantity and quality, amino acid or protein composition, or those increasing yield or nutritional quality may be employed as desired.

The construction of vectors which may be employed in conjunction with plant transformation techniques according to the invention will be known to those of skill of the art in light of the present disclosure (see for example, Sambrook *et al.*, 1989; Gelvin *et al.*, 1990). The techniques of the current invention are thus not limited to any particular DNA sequences in conjunction with the actin 2 intron and/or actin 2 promoter of the invention. For example, the actin 2 intron, actin 2 promoter, or actin 2 promoter and intron could be transformed into a plant with the goal of enhancing or altering the expression of one or more genes in the host genome.

In certain embodiments of the invention, it is contemplated that one may wish to employ replication-competent viral vectors for plant transformation. Such vectors include, for example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and PW1-GUS (Ugaki *et al.*, 1991). These vectors are capable of autonomous replication in maize cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector also may be useful for delivery of genes flanked by DNA sequences from transposable elements such as Ac, Ds, or Mu. It has been proposed that transposition of these elements within the maize genome requires DNA replication (Laufs *et al.*, 1990). It also is contemplated that transposable elements would be useful for introducing DNA fragments lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, *e.g.*, antibiotic resistance genes and origins of DNA replication. It also is proposed that use of a transposable element such as Ac, Ds, or Mu would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells. It also is proposed that transposable elements would be useful to allow separation of genes of interest from elements necessary for selection and maintenance of a plasmid vector in bacteria or selection of a transformant. By use of a transposable element, desirable and undesirable DNA sequences may be transposed apart from each other in the genome, such that through genetic segregation in progeny, one may identify plants with either the desirable undesirable DNA sequences.

It further is contemplated that one may wish to co-transform plants or plant cells with 2 or more vectors. Co-transformation may be achieved using a vector containing the marker and

another gene or genes of interest. Alternatively, different vectors, *e.g.*, plasmids, may contain the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. Using this method, the assumption is made that a certain percentage of cells in which the marker has been introduced, also have received the other gene(s) of interest. Thus, not all cells selected
5 by means of the marker, will express the other genes of interest which had been presented to the cells concurrently.

Vectors used for plant transformation may include, for example, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) or other cloning systems. Particularly useful for transformation are expression cassettes which have been isolated from such
10 vectors. DNA segments used for transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduced into and have expressed in the host cells. These DNA segments can further include, in addition to an actin 2 intron or actin 2 promoter, structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be
15 expressed in the resultant recombinant cells resulting in a screenable or selectable trait and/or which will impart an improved phenotype to the resulting transgenic plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

20 (i) *Regulatory Elements*

Constructs prepared in accordance with the current invention will include an actin 2 intron and/or an actin 2 promoter. However, these elements may be used in the preparation of transformation constructs which comprise a wide variety of other elements. One such application in accordance with the instant invention will be the preparation of transformation constructs
25 comprising the actin 2 intron operably linked to a plant promoter other than the actin 2 promoter.

A promoter is selected for it's ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Useful plant promoters include those that are inducible, viral, synthetic, constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989).

Exemplary constitutive promoters include the CaMV 35S promoter (Odell *et al.*, 1985), CaMV 19S (Lawton *et al.*, 1987), actin (Wang *et al.*, 1992), and *nos* (Ebert *et al.*, 1987). Where the promoter is a near-constitutive promoter, increases in polypeptide expression generally are found in a variety of transformed plant tissues (*e.g.*, callus, leaf, seed and root).

5 Exemplary tissue-specific promoters include lectin (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989; Dennis *et al.*, 1984); corn light harvesting complex (Simpson, 1986; Bansal *et al.*, 1992), corn heat shock protein (Odell *et al.*, 1985; Rochester *et al.*, 1986), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid
10 nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), truncated CaMV 35s (Odell *et al.*, 1985), potato patatin promoters (Wenzler *et al.*, 1989), root cell promoters (Conkling *et al.*, 1990), tissue specific enhancers (Fromm *et al.*, 1989), maize zein (Reina *et al.*, 1990; Kriz *et al.*, 1987; Wandelt and Feix, 1989; Langridge and Feix, 1983; Reina *et al.*, 1990), globulin-1 (Belanger &
15 Kriz, 1991), Adh (Walker *et al.*, 1987), histone, sucrose synthase (Yang & Russell, 1990), α -tubulin, cab (Sullivan *et al.*, 1989), PEPCase (Hudspeth & Grula, 1989) and R gene complex-associated promoters (Chandler *et al.*, 1989) and chalcone synthase (Franken *et al.*, 1991).

Examples of inducible promoters include ABA- and turgor-inducible promoters and the promoter of the auxin-binding protein gene (Scwob *et al.*, 1993; Genbank Accession No.
20 L08425). Still other potentially useful promoters include the UDP glucose flavonoid glycosyl-transferase gene promoter (Ralston *et al.*, 1988); MPI proteinase inhibitor (Cordero *et al.*, 1994), and the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler *et al.*, 1995; Quigley *et al.*, 1989; Martinez *et al.*, 1989), as well as promoters of chloroplast genes (Genbank Accession No. X86563).

25 In addition to promoters, other types of elements can regulate gene expression. One such element which could be used in conjunction with the rice actin 2 intron and/or the rice actin 2 promoter of the instant invention is the DNA sequence between the transcription initiation site and the start of the coding sequence, termed the untranslated leader sequence. The leader sequence can influence gene expression and compilations of leader sequences have been made to
30 predict optimum or sub-optimum sequences and generate "consensus" and preferred leader

sequences (Joshi, 1987). Preferred leader sequences are contemplated to include those which have sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred.

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. In some instances these 5' enhancing elements are introns. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin 1 gene, the maize alcohol dehydrogenase gene (Callis *et al.*, 1987), the maize shrunken 1 gene (Vasil *et al.*, 1989), TMV Omega element (Gallie *et al.*, 1989) and promoters from non-plant eukaryotes (*e.g.* yeast; Ma *et al.*, 1988).

Specifically contemplated for use in accordance with the present invention are vectors which include the ocs enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (ocs) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the ocs element and particularly multiple copies of the element, may be used to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation.

It is contemplated that introduction of large DNA sequences comprising more than one gene may be desirable. Introduction of such sequences may be facilitated by use of bacterial or yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes. For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Ultimately, the most desirable DNA segments for introduction into a plant genome may be homologous genes or gene families which encode a desired trait, and which are introduced under the control of novel promoters or enhancers, for example, the rice actin 2 promoter or rice

actin 2 intron. Tissue specific regulatory regions may be particularly useful in conjunction with the actin 2 intron. Indeed, it is envisioned that a particular use of the present invention may be the production of transformants comprising a transgene which is expressed in a tissue-specific manner, whereby the expression is enhanced by the actin 2 intron. For example, insect resistant genes may be expressed specifically in the whorl and collar/sheath tissues which are targets for the first and second broods, respectively, of European Corn Borer (ECB). Likewise, genes encoding proteins with particular activity against rootworm may be targeted directly to root tissues. In addition, expression of certain genes which affect the nutritional composition of the grain must be targeted to the seed, *e.g.*, endosperm or embryo.

Vectors for use in tissue-specific targeting of gene expression in transgenic plants typically will include tissue-specific promoters and also may include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues in accordance with the invention will be known to those of skill in the art in light of the present disclosure.

It also is contemplated that tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for the crystal toxin protein from *B. thuringiensis* (Bt) may be introduced such that it is expressed in all tissues using a constitutive promoter, for example with a rice actin 2 promoter. Therefore, expression of an antisense transcript of the Bt gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the Bt protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel. Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, U.S. Patent No. 5,491,288 discloses combining a Cauliflower Mosaic Virus promoter with a histone promoter.

Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use with the rice actin 2 intron of the current invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific tissues.

The promoter and control elements of corresponding genomic clones may then be localized using the techniques of molecular biology known to those of skill in the art.

Another useful method for identifying tissue-specific promoters is differential display (see, e.g., U.S. Patent No. 5,599,672, the disclosure of which is specifically incorporated herein by reference in its entirety). In differential display, mRNAs are compared from different tissue types. By identifying mRNA species which are present in only a particular tissue type, or set of tissues types, one can identify the corresponding genes which are expressed in a tissue specific manner. The RNAs can be transcribed by reverse transcriptase to produce a cDNA, and the cDNA in turn be used to isolate clones containing the full-length genes. As specifically disclosed herein, the cDNA also can be used to isolate homeologous or homologous promoters, enhancers or terminators from the respective gene using, for example, suppression PCR.

It is contemplated that expression of some genes in transgenic plants will be desired only under specified conditions. For example, it is proposed that expression of certain genes that confer resistance to environmental stress factors such as drought will be desired only under actual stress conditions. It further is contemplated that expression of such genes throughout a plant's development may have detrimental effects. It is known that a large number of genes exist that respond to the environment. For example, expression of some genes such as *rbcS*, encoding the small subunit of ribulose biphosphate carboxylase, are regulated by light as mediated through phytochrome. Other genes are induced by secondary stimuli. For example, synthesis of abscisic acid (ABA) is induced by certain environmental factors, including but not limited to water stress. A number of genes have been shown to be induced by ABA (Skriver and Mundy, 1990). It also is anticipated that expression of genes conferring resistance to insect predation would be desired only under conditions of actual insect infestation. Therefore, for some desired traits, inducible expression of genes in transgenic plants will be desired.

It is proposed that, in some embodiments of the present invention, expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing frequently is correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated in the endosperm about 10 days after pollination.

It also is contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful to target a gene in order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation replace an existing gene in the cell.

(ii) *Terminators*

Constructs will typically include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as Adh intron (Callis *et al.*, 1987), sucrose synthase intron (Vasil *et al.*, 1989) or TMV omega element (Gallie, *et al.*, 1989), may further be included where desired. Alternatively, one also could use a gamma coixin, oleosin 3 or other terminator from the genus *Coix*.

(iii) *Transit or Signal Peptides*

Sequences that are joined to the coding sequence of an expressed gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes, are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the endoplasmic reticulum, golgi apparatus and outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It is further contemplated that targeting

of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed.

A particular example of such a use concerns the direction of a protein conferring herbicide resistance, such as a mutant EPSPS protein, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the *rbcS* transit peptide, the chloroplast transit peptide described in U.S. Patent No. 5,728,925, or the optimized transit peptide described in U.S. Patent No. 5,510,471, which confer plastid-specific targeting of proteins. In addition, it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole. A further use concerns the direction of enzymes involved in amino acid biosynthesis or oil synthesis to the plastid. Such enzymes include dihydrodipicolinic acid synthase which may contribute to increasing lysine content of a feed.

(iv) *Marker Genes*

One important application of the rice actin 2 intron and/or rice actin 2 promoter of the current invention will be in the expression of marker genes. By employing a selectable or screenable marker gene as, or in addition to, the expressible gene of interest, one can provide or enhance the ability to identify transformants. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, *i.e.*, by

"screening" (e.g., the green fluorescent protein). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes also are genes which
5 encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; small active enzymes detectable in extracellular solution
10 (e.g., α -amylase, β -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that
15 becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall
20 protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of maize HPRG (Steifel *et al.*, 1990) is preferred, as this molecule is well characterized in terms of molecular biology, expression and
25 protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller *et al.*, 1989) could be modified by the addition of an antigenic site to create a screenable marker.

One exemplary embodiment of a secretable screenable marker concerns the use of a maize
30 sequence encoding the wall protein HPRG, modified to include a 15 residue epitope from the pro-region of murine interleukin-1- β (IL-1- β). However, virtually any detectable epitope may be employed in such embodiments, as selected from the extremely wide variety of antigen:antibody

combinations known to those of skill in the art. The unique extracellular epitope, whether derived from IL-1 β or any other protein or epitopic substance, can then be straightforwardly detected using antibody labeling in conjunction with chromogenic or fluorescent adjuncts.

5 1. Selectable Markers

Many selectable marker genes may be used in connection with the actin 2 intron and/or actin 2 promoter of the present invention including, but not limited to, a *neo* gene (Potrykus *et al.*, 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; a *bar* gene which confers bialaphos or phosphinothricin resistance; a
10 glyphosate resistant EPSP synthase protein (Hinchee *et al.*, 1988); a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR gene (Thillet *et al.*, 1988), a dalapon dehalogenase gene that confers resistance to
15 the herbicide dalapon (U.S. Patent No. 5,980,708); or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan (WO 97/26366; U.S. Patent No. 4,581,847). Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (U.S. Patent No. 5,188,642) or OTP (U.S. Patent No. 5,633,448) and use of a modified maize EPSPS gene (PCT Application WO
20 97/04103).

An illustrative embodiment of selectable marker genes capable of being used in systems to select transformants are the genes that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces*
25 *viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.*, 1986; Twell *et al.*, 1989) causing rapid accumulation of ammonia and cell death.

30 Where one desires to employ a bialaphos resistance gene in the practice of the invention, the inventor has discovered that particularly useful genes for this purpose are the *bar* or *pat* genes obtainable from species of *Streptomyces* (*e.g.*, ATCC No. 21,705). The cloning of the *bar* gene has

been described (Murakami *et al.*, 1986; Thompson *et al.*, 1987) as has the use of the *bar* gene in the context of plants (De Block *et al.*, 1987; De Block *et al.*, 1989; U.S. Patent No. 5,550,318).

2. Screenable Markers

5 Screenable markers that may be employed include a β -glucuronidase (GUS) or *uidA* gene which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a β -lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic
10 cephalosporin); a *xyIE* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α -amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a
15 luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; WO 97/41228).

20 Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. Thus, an R gene introduced into such cells will cause the expression of a red
25 pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding for the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the *rg*-Stadler allele and TR112, a K55 derivative
30 which is *r-g, b, Pl*. Alternatively, any genotype of maize can be utilized if the C1 and R alleles are introduced together.

It is further proposed that R gene regulatory regions may be employed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe *et al.*, 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes for, *e.g.*, insect resistance, herbicide tolerance or other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (*e.g.*, P, S, Lc, *etc.*). However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein (GFP) is contemplated as a particularly useful reporter gene (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; WO 97/41228). Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light. Where use of a screenable marker gene such as *lux* or GFP is desired, the inventors contemplated that benefit may be realized by creating a gene fusion between the screenable marker gene and a selectable marker gene, for example, a GFP-NPTII gene fusion. This could allow, for example, selection of transformed cells followed by screening of transgenic plants or seeds.

II. Exogenous Genes for Modification of Plant Phenotypes

A particularly important advance of the present invention is that it provides methods and compositions for the efficient expression in plant cells of genes in addition to, or other than, marker genes. Such transgenes often will be genes that direct the expression of a particular protein or polypeptide product, but they also may be non-expressible DNA segments, *e.g.*,

transposons such as Ds that do not direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (*e.g.*, mRNA, antisense RNA, *etc.*) or translated into a protein, expressed as a trait of interest, or the like, *etc.*, and is not limited to selectable, screenable or non-selectable marker genes. The inventors also
5 contemplate that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

10 The choice of the particular DNA segments to be delivered to the recipient cells often will depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance
15 and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch quantity and quality; oil quantity and quality; protein quality and quantity; amino acid composition; and the like. One may desire to incorporate one or more genes conferring any such desirable trait or traits, such as,
20 for example, a gene or genes encoding herbicide resistance.

In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one exogenous gene. As used herein, an "exogenous gene," is a gene not normally found in the host genome in an identical context. By this, it is meant that the
25 gene may be isolated from a different species than that of the host genome, or alternatively, isolated from the host genome but operably linked to one or more regulatory regions which differ from those found in the unaltered, native gene. Two or more exogenous genes also can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids
30 bearing the *bar* and *aroA* expression units in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect resistance gene, such as a Bt gene, along with a protease inhibitor gene such as *pinII*, or the use

of *bar* in combination with either of the above genes. Of course, any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

(i) *Herbicide Resistance*

The genes encoding phosphinothricin acetyltransferase (*bar* and *pat*), glyphosate tolerant EPSP synthase genes, the glyphosate degradative enzyme gene *gox* encoding glyphosate oxidoreductase, *deh* (encoding a dehalogenase enzyme that inactivates dalapon), herbicide resistant (*e.g.*, sulfonylurea and imidazolinone) acetolactate synthase, and *bxn* genes (encoding a nitrilase enzyme that degrades bromoxynil) are good examples of herbicide resistant genes for use in transformation. The *bar* and *pat* genes code for an enzyme, phosphinothricin acetyltransferase (PAT), which inactivates the herbicide phosphinothricin and prevents this compound from inhibiting glutamine synthetase enzymes. The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP Synthase), is normally inhibited by the herbicide N-(phosphonomethyl)glycine (glyphosate). However, genes are known that encode glyphosate-resistant EPSP synthase enzymes. These genes are particularly contemplated for use in plant transformation. The *deh* gene encodes the enzyme dalapon dehalogenase and confers resistance to the herbicide dalapon. The *bxn* gene codes for a specific nitrilase enzyme that converts bromoxynil to a non-herbicidal degradation product.

(ii) *Insect Resistance*

Potential insect resistance genes that can be introduced include *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Bt genes may provide resistance to lepidopteran or coleopteran pests such as European Corn Borer (ECB) and Corn Rootworm (CRW). Preferred Bt toxin genes for use in such embodiments include the *CryIA(b)* and *CryIA(c)* genes. Endotoxin genes from other species of *B. thuringiensis* which affect insect growth or development also may be employed in this regard.

It is contemplated that preferred Bt genes for use in the transformation protocols disclosed herein will be those in which the coding sequence has been modified to effect increased

expression in plants, and more particularly, in maize. Means for preparing synthetic genes are well known in the art and are disclosed in, for example, U.S. Patent No. 5,500,365 and U.S. Patent Number No. 5,689,052, each of the disclosures of which are specifically incorporated herein by reference in their entirety. Examples of such modified Bt toxin genes include a
5 synthetic Bt *CryIA(b)* gene (Perlak *et al.*, 1991), and the synthetic *CryIA(c)* gene termed 1800b (PCT Application WO 95/06128). Some examples of other Bt toxin genes known to those of skill in the art are given in Table 1 below.

Table 1

10 *Bacillus thuringiensis* δ -Endotoxin Genes^a

New Nomenclature	Old Nomenclature	GenBank Accession
CryIAa	CryIA(a)	M11250
CryIAb	CryIA(b)	M13898
CryIAc	CryIA(c)	M11068

Table 1 - Continued
***Bacillus thuringiensis* δ -Endotoxin Genes^a**

New Nomenclature	Old Nomenclature	GenBank Accession
Cry1Ad	CryIA(d)	M73250
Cry1Ae	CryIA(e)	M65252
Cry1Ba	CryIB	X06711
Cry1Bb	ET5	L32020
Cry1Bc	PEG5	Z46442
Cry1Bd	CryE1	U70726
Cry1Ca	CryIC	X07518
Cry1Cb	CryIC(b)	M97880
Cry1Da	CryID	X54160
Cry1Db	PrtB	Z22511
Cry1Ea	CryIE	X53985
Cry1Eb	CryIE(b)	M73253
Cry1Fa	CryIF	M63897
Cry1Fb	PrtD	Z22512
Cry1Ga	PrtA	Z22510
Cry1Gb	CryH2	U70725
Cry1Ha	PrtC	Z22513
Cry1Hb		U35780
CryIIa	CryV	X62821
CryIIb	CryV	U07642
Cry1Ja	ET4	L32019
Cry1Jb	ET1	U31527
Cry1K		U28801
Cry2Aa	CryIIA	M31738
Cry2Ab	CryIIB	M23724
Cry2Ac	CryIIC	X57252
Cry3A	CryIIIA	M22472

Table 1 - Continued
Bacillus thuringiensis δ -Endotoxin Genes^a

New Nomenclature	Old Nomenclature	GenBank Accession
Cry3Ba	CryIIIB	X17123
Cry3Bb	CryIIIB2	M89794
Cry3C	CryIIID	X59797
Cry4A	CryIVA	Y00423
Cry4B	CryIVB	X07423
Cry5Aa	CryVA(a)	L07025
Cry5Ab	CryVA(b)	L07026
Cry6A	CryVIA	L07022
Cry6B	CryVIB	L07024
Cry7Aa	CryIIIC	M64478
Cry7Ab	CryIIICb	U04367
Cry8A	CryIIIE	U04364
Cry8B	CryIIIG	U04365
Cry8C	CryIIIF	U04366
Cry9A	CryIG	X58120
Cry9B	CryIX	X75019
Cry9C	CryIH	Z37527
Cry10A	CryIVC	M12662
Cry11A	CryIVD	M31737
Cry11B	Jeg80	X86902
Cry12A	CryVB	L07027
Cry13A	CryVC	L07023
Cry14A	CryVD	U13955
Cry15A	34kDa	M76442
Cry16A	cbm71	X94146
Cry17A	cbm71	X99478

Table 1 - Continued
Bacillus thuringiensis δ -Endotoxin Genes*

New Nomenclature	Old Nomenclature	GenBank Accession
Cry18A	CryBP1	X99049
Cry19A	Jeg65	Y08920
Cyt1Aa	CytA	X03182
Cyt1Ab	CytM	X98793
Cyt2A	CytB	Z14147
Cyt2B	CytB	U52043

*Adapted from: http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html

5 Protease inhibitors also may provide insect resistance (Johnson *et al.*, 1989), and will thus have utility in plant transformation. The use of a protease inhibitor II gene, *pinII*, from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a *pinII* gene in combination with a Bt toxin gene, the combined effect of which has been discovered to produce synergistic insecticidal activity. Other genes which encode inhibitors of the insect's
 10 digestive system, or those that encode enzymes or co-factors that facilitate the production of inhibitors, also may be useful. This group may be exemplified by oryzacystatin and amylase inhibitors such as those from wheat and barley.

Also, genes encoding lectins may confer additional or alternative insecticide properties.
 15 Lectins (originally termed phytohemagglutinins) are multivalent carbohydrate-binding proteins which have the ability to agglutinate red blood cells from a range of species. Lectins have been identified recently as insecticidal agents with activity against weevils, ECB and corn rootworm (Murdock *et al.*, 1990; Czapla & Lang, 1990). Lectin genes contemplated to be useful include, for example, barley and wheat germ agglutinin (WGA) and rice lectins (Gatehouse *et al.*, 1984),
 20 with WGA being preferred.

Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as, *e.g.*, lytic peptides, peptide hormones and toxins and venoms, form another aspect of the invention. For example, it is contemplated that the

expression of juvenile hormone esterase, directed towards specific insect pests, also may result in insecticidal activity, or perhaps cause cessation of metamorphosis (Hammock *et al.*, 1990).

Transgenic plants expressing genes which encode enzymes that affect the integrity of the insect cuticle form yet another aspect of the invention. Such genes include those encoding, *e.g.*, chitinase, proteases, lipases and also genes for the production of nikkomycin, a compound that inhibits chitin synthesis, the introduction of any of which is contemplated to produce insect resistant plants. Genes that code for activities that affect insect molting, such as those affecting the production of ecdysteroid UDP-glucosyl transferase, also fall within the scope of the useful transgenes of the present invention.

Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests also are encompassed by the present invention. It may be possible, for instance, to confer insecticidal activity on a plant by altering its sterol composition. Sterols are obtained by insects from their diet and are used for hormone synthesis and membrane stability. Therefore alterations in plant sterol composition by expression of novel genes, *e.g.*, those that directly promote the production of undesirable sterols or those that convert desirable sterols into undesirable forms, could have a negative effect on insect growth and/or development and hence endow the plant with insecticidal activity. Lipxygenases are naturally occurring plant enzymes that have been shown to exhibit anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Therefore, further embodiments of the invention concern transgenic plants with enhanced lipxygenase activity which may be resistant to insect feeding.

Tripsacum dactyloides is a species of grass that is resistant to certain insects, including corn root worm. It is anticipated that genes encoding proteins that are toxic to insects or are involved in the biosynthesis of compounds toxic to insects will be isolated from *Tripsacum* and that these novel genes will be useful in conferring resistance to insects. It is known that the basis of insect resistance in *Tripsacum* is genetic, because said resistance has been transferred to *Zea mays* via sexual crosses (Branson and Guss, 1972). It is further anticipated that other cereal, monocot or dicot plant species may have genes encoding proteins that are toxic to insects which would be useful for producing insect resistant corn plants.

Further genes encoding proteins characterized as having potential insecticidal activity also may be used as transgenes in accordance herewith. Such genes include, for example, the cowpea trypsin inhibitor (CpTI; Hilder *et al.*, 1987) which may be used as a rootworm deterrent; genes
5 encoding avermectin (*Avermectin and Abamectin.*, Campbell, W.C., Ed., 1989; Ikeda *et al.*, 1987) which may prove particularly useful as a corn rootworm deterrent; ribosome inactivating protein genes; and even genes that regulate plant structures. Transgenic maize including anti-insect antibody genes and genes that code for enzymes that can convert a non-toxic insecticide (pro-insecticide) applied to the outside of the plant into an insecticide inside the plant also are
10 contemplated.

(iii). *Environment or Stress Resistance*

Improvement of a plants ability to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative
15 stress, also can be effected through expression of novel genes. It is proposed that benefits may be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein such as that of the Winter Flounder (Cutler *et al.*, 1989) or synthetic gene derivatives thereof. Improved chilling tolerance also may be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Wolter *et al.*, 1992).
20 Resistance to oxidative stress (often exacerbated by conditions such as chilling temperatures in combination with high light intensities) can be conferred by expression of superoxide dismutase (Gupta *et al.*, 1993), and may be improved by glutathione reductase (Bowler *et al.*, 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity zones.

25

It is contemplated that the expression of novel genes that favorably effect plant water content, total water potential, osmotic potential, and turgor will enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in
30 water availability, as compared to normal circumstances, and the ability of the plant to function and survive in lower-water environments. In this aspect of the invention it is proposed, for example, that the expression of genes encoding for the biosynthesis of osmotically-active solutes,

such as polyol compounds, may impart protection against drought. Within this class are genes encoding for mannitol-1-phosphate dehydrogenase (Lee and Saier, 1982) and trehalose-6-phosphate synthase (Kaasen *et al.*, 1992). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these introduced
5 genes will result in the accumulation of either mannitol or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski *et al.*, 1992, 1993). Furthermore, expression of mannitol-1-phosphate
10 dehydrogenase in corn and correlation of expression with accumulation of mannitol and altered water utilization have been demonstrated (U.S. Patent No. 5,780,709).

Similarly, the efficacy of other metabolites in protecting either enzyme function (*e.g.*, alanopine or propionic acid) or membrane integrity (*e.g.*, alanopine) has been documented
15 (Loomis *et al.*, 1989), and therefore expression of genes encoding for the biosynthesis of these compounds might confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include fructose, erythritol (Coxson *et al.*, 1992), sorbitol, dulcitol (Karsten *et al.*, 1992), glucosylglycerol (Reed *et al.*,
20 1984; Erdmann *et al.*, 1992), sucrose, stachyose (Koster and Leopold, 1988; Blackman *et al.*, 1992), raffinose (Bernal-Lugo and Leopold, 1992), proline (Rensburg *et al.*, 1993), glycine betaine, ononitol and pinitol (Vernon and Bohnert, 1992). Continued canopy growth and increased reproductive fitness during times of stress will be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above
25 and other such compounds. Currently preferred genes which promote the synthesis of an osmotically active polyol compound are genes which encode the enzymes mannitol-1-phosphate dehydrogenase, trehalose-6-phosphate synthase and myoinositol 0-methyltransferase.

It is contemplated that the expression of specific proteins also may increase drought
30 tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure *et al.*, 1989). All three classes of LEAs have been demonstrated in maturing (*i.e.* desiccating) seeds. Within these 3 types of LEA proteins, the Type-II (dehydrin-

type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (*i.e.* Mundy and Chua, 1988; Piatkowski *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and drought tolerance (Fitzpatrick, 1993). In rice, expression of the HVA-1 gene
5 influenced tolerance to water deficit and salinity (Xu *et al.*, 1996). Expression of structural genes from all three LEA groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero *et al.*, 1990), which may confer various protective and/or repair-type functions during drought stress. It also is contemplated that genes that effect lipid biosynthesis and hence
10 membrane composition might also be useful in conferring drought resistance on the plant.

Many of these genes for improving drought resistance have complementary modes of action. Thus, it is envisaged that combinations of these genes might have additive and/or synergistic effects in improving drought resistance in corn. Many of these genes also improve
15 freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be mitigated in similar fashion. Benefit may be conferred via constitutive expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero *et al.*, 1990 and Shagan *et al.*, 1993 which are incorporated
20 herein by reference). Spatial and temporal expression patterns of these genes may enable plants to better withstand stress.

It is proposed that expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example,
25 introduction and expression of genes that alter root characteristics may enhance water uptake. It also is contemplated that expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of genes that improve the synchrony of pollen shed and receptiveness of the female flower parts, *i.e.*, silks, would be of benefit. In addition it is proposed that expression of genes that minimize kernel abortion during
30 times of stress would increase the amount of grain to be harvested and hence be of value.

Given the overall role of water in determining yield, it is contemplated that enabling corn to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of corn to maximize water usage across a full range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

(iv) *Disease Resistance*

It is proposed that increased resistance to diseases may be realized through introduction of genes into plants, for example, into monocotyledonous plants such as maize. It is possible to produce resistance to diseases caused by viruses, bacteria, fungi and nematodes. It also is contemplated that control of mycotoxin producing organisms may be realized through expression of introduced genes.

Resistance to viruses may be produced through expression of novel genes. For example, it has been demonstrated that expression of a viral coat protein in a transgenic plant can impart resistance to infection of the plant by that virus and perhaps other closely related viruses (Cuozzo *et al.*, 1988, Hemenway *et al.*, 1988, Abel *et al.*, 1986). It is contemplated that expression of antisense genes targeted at essential viral functions may also impart resistance to viruses. For example, an antisense gene targeted at the gene responsible for replication of viral nucleic acid may inhibit replication and lead to resistance to the virus. It is believed that interference with other viral functions through the use of antisense genes also may increase resistance to viruses. Further, it is proposed that it may be possible to achieve resistance to viruses through other approaches, including, but not limited to the use of satellite viruses. Examples of viral and viral-like diseases, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Table 2.

Table 2

Plant Virus and Virus-like Diseases

DISEASE	CAUSATIVE AGENT
American wheat striate (wheat striate mosaic)	American wheat striate mosaic virus mosaic (AWSMV)
Barley stripe mosaic	Barley stripe mosaic virus (BSMV)

DISEASE	CAUSATIVE AGENT
Barley yellow dwarf	Barley yellow dwarf virus (BYDV)
Brome mosaic	Brome mosaic virus (BMV)
Cereal chlorotic mottle*	Cereal chlorotic mottle virus (CCMV)
Corn chlorotic vein banding (Brazilian maize mosaic) ¹	Corn chlorotic vein banding virus (CCVBV)
Corn lethal necrosis	Virus complex (Maize chlorotic mottle virus(MCMV) and Maize dwarf mosaic virus (MDMV) A or B or Wheat streak mosaic virus (WSMV))
Cucumber mosaic	Cucumber mosaic virus (CMV)
Cynodon chlorotic streak** ¹	Cynodon chlorotic streak virus (CCSV)
Johnsongrass mosaic	Johnsongrass mosaic virus (JGMV)
Maize bushy stunt	Mycoplasma-like organism (MLO) associated
Maize chlorotic dwarf	Maize chlorotic dwarf virus (MCDV)
Maize chlorotic mottle	Maize chlorotic mottle virus (MCMV)
Maize dwarf mosaic	Maize dwarf mosaic virus (MDMV) strains A, D, E and F
Maize leaf fleck	Maize leaf fleck virus (MLFV)
Maize line*	Maize line virus (MLV)
Maize mosaic (corn leaf stripe, enanismo rayado)	Maize mosaic virus (MMV)
Maize mottle and chlorotic stunt ¹	Maize mottle and chlorotic stunt virus*
Maize pellucid ringspot*	Maize pellucid ringspot virus (MPRV)
Maize raya gruesa** ¹	Maize raya gruesa virus (MRGV)

Table 2 - Continued
Plant Virus and Virus-like Diseases

DISEASE	CAUSATIVE AGENT
maize rayado fino* (fine striping disease)	Maize rayado fino virus (MRFV)
Maize red leaf and red stripe*	Mollicute?
Maize red stripe*	Maize red stripe virus (MRSV)
Maize ring mottle*	Maize ring mottle virus (MRMV)
Maize rio IV*	Maize rio cuarto virus (MRCV)
Maize rough dwarf* (nanismo ruvido)	Maize rough dwarf virus (MRDV) (= Cereal tillering disease virus*)
Maize sterile stunt*	Maize sterile stunt virus (strains of barley yellow striate virus)
Maize streak*	Maize streak virus (MSV)
Maize stripe (maize chlorotic stripe, maize hoja blanca)	Maize stripe virus
Maize stunting*, ¹	Maize stunting virus
Maize tassel abortion*	Maize tassel abortion virus (MTAV)
Maize vein enation*	Maize vein enation virus (MVEV)
Maize wallaby ear*	Maize wallaby ear virus (MWEV)
Maize white leaf*	Maize white leaf virus
Maize white line mosaic	Maize white line mosaic virus (MWLMV)
Millet red leaf*	Millet red leaf virus (MRLV)
Northern cereal mosaic*	Northern cereal mosaic virus (NCMV)
Oat pseudorosette* (zakuklivanie)	Oat pseudorosette virus
Oat sterile dwarf*	Oat sterile dwarf virus (OSDV)
Rice black-streaked dwarf*	Rice black-streaked dwarf virus (RBSDV)
Rice stripe*	Rice stripe virus (RSV)

Table 2 - Continued
Plant Virus and Virus-like Diseases

DISEASE	CAUSATIVE AGENT
Sorghum mosaic	Sorghum mosaic virus (SrMV), formerly sugarcane mosaic virus (SCMV) strains H, I and M
Sugarcane Fiji disease*	Sugarcane Fiji disease virus (FDV)
Sugarcane mosaic	Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B)
Vein enation*, ¹	Virus ?
Wheat spot mosaic ¹	Wheat spot mosaic virus (WSMV)

*Not known to occur naturally on corn in the United States. ¹ Minor viral disease.

- 5 It is proposed that increased resistance to diseases caused by bacteria and fungi may be realized through introduction of novel genes. It is contemplated that genes encoding so-called "peptide antibiotics," pathogenesis related (PR) proteins, toxin resistance, and proteins affecting host-pathogen interactions such as morphological characteristics will be useful. Peptide antibiotics are polypeptide sequences which are inhibitory to growth of bacteria and other
- 10 microorganisms. For example, the classes of peptides referred to as cecropins and magainins inhibit growth of many species of bacteria and fungi. It is proposed that expression of PR proteins in monocotyledonous plants such as maize may be useful in conferring resistance to bacterial disease. These genes are induced following pathogen attack on a host plant and have been divided into at least five classes of proteins (Bol, Linthorst, and Cornelissen, 1990).
- 15 Included amongst the PR proteins are β -1, 3-glucanases, chitinases, and osmotin and other proteins that are believed to function in plant resistance to disease organisms. Other genes have been identified that have antifungal properties, *e.g.*, UDA (stinging nettle lectin) and hevein (Broekaert *et al.*, 1989; Barkai-Golan *et al.*, 1978). It is known that certain plant diseases are caused by the production of phytotoxins. It is proposed that resistance to these diseases would
- 20 be achieved through expression of a novel gene that encodes an enzyme capable of degrading or otherwise inactivating the phytotoxin. It also is contemplated that expression of novel genes that alter the interactions between the host plant and pathogen may be useful in reducing the ability of

the disease organism to invade the tissues of the host plant, *e.g.*, an increase in the waxiness of the leaf cuticle or other morphological characteristics. Examples of bacterial and fungal diseases, including downy mildews, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Tables 3, 4 and 5.

5

Table 3
Plant Bacterial Diseases

DISEASE	CAUSATIVE AGENT
Bacterial leaf blight and stalk rot	<i>Pseudomonas avenae</i> subsp. <i>avenae</i>
Bacterial leaf spot	<i>Xanthomonas campestris</i> pv. <i>holcicola</i>
Bacterial stalk rot	<i>Enterobacter dissolvens</i> = <i>Erwinia dissolvens</i>
Bacterial stalk and top rot	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> , <i>Erwinia chrysanthemi</i> pv. <i>zeae</i>
Bacterial stripe	<i>Pseudomonas andropogonis</i>
Chocolate spot	<i>Pseudomonas syringae</i> pv. <i>coronafaciens</i>
Goss's bacterial wilt and blight (leaf freckles and wilt)	<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i> = <i>Corynebacterium michiganense</i> pv. <i>nebraskense</i>
Holcus spot	<i>Pseudomonas syringae</i> pv. <i>syringae</i>
Purple leaf sheath	Hemiparasitic bacteria + (See under Fungi)
Seed rot-seedling blight	<i>Bacillus subtilis</i>
Stewart's disease (bacterial wilt)	<i>Pantoea stewartii</i> = <i>Erwinia stewartii</i>
Corn stunt (achapparramiento, maize stunt, Mesa Central or Rio Grande maize stunt)	<i>Spiroplasma kunkelii</i>

Table 4
Plant Fungal Diseases

DISEASE	PATHOGEN
Anthracnose leaf blight and anthracnose stalk rot	<i>Colletotrichum graminicola</i> (teleomorph: <i>Glomerella graminicola</i> Politis), <i>Glomerella tucumanensis</i> (anamorph: <i>Glomerella falcatum</i> Went)
Aspergillus ear and kernel rot	<i>Aspergillus flavus</i> Link:Fr.
Banded leaf and sheath spot*	<i>Rhizoctonia solani</i> Kühn = <i>Rhizoctonia microsclerotia</i> J. Matz (teleomorph: <i>Thanatephorus cucumeris</i>)
Black bundle disease	<i>Acremonium strictum</i> W. Gams = <i>Cephalosporium acremonium</i> Auct. non Corda
Black kernel rot*	<i>Lasiodiplodia theobromae</i> = <i>Botryodiplodia theobromae</i>
Borde blanco*	<i>Marasmiellus</i> sp.
Brown spot (black spot, stalk rot)	<i>Physoderma maydis</i>
Cephalosporium kernel rot	<i>Acremonium strictum</i> = <i>Cephalosporium acremonium</i>
Charcoal rot	<i>Macrophomina phaseolina</i>
Corticium ear rot*	<i>Thanatephorus cucumeris</i> = <i>Corticium sasakii</i>

Table 4 - Continued
Plant Fungal Diseases

DISEASE	PATHOGEN
Curvularia leaf spot	<i>Curvularia clavata</i> , <i>C. eragrostidis</i> , = <i>C. maculans</i> (teleomorph: <i>Cochliobolus eragrostidis</i>), <i>Curvularia inaequalis</i> , <i>C. intermedia</i> (teleomorph: <i>Cochliobolus intermedius</i>), <i>Curvularia lunata</i> (teleomorph: <i>Cochliobolus lunatus</i>), <i>Curvularia pallescens</i> (teleomorph: <i>Cochliobolus pallescens</i>), <i>Curvularia senegalensis</i> , <i>C. tuberculata</i> (teleomorph: <i>Cochliobolus tuberculatus</i>)
Didymella leaf spot*	<i>Didymella exitalis</i>
Diplodia ear rot and stalk rot	<i>Diplodia frumenti</i> (teleomorph: <i>Botryosphaeria festucae</i>)
Diplodia ear rot, stalk rot, seed rot and seedling blight	<i>Diplodia maydis</i> = <i>Stenocarpella maydis</i>
Diplodia leaf spot or leaf streak	<i>Stenocarpella macrospora</i> = <i>Diplodia macrospora</i>

*Not known to occur naturally on corn in the United States.

Table 5
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Brown stripe downy mildew*	<i>Sclerophthora rayssiae</i> var. <i>zeae</i>
Crazy top downy mildew	<i>Sclerophthora macrospora</i> = <i>Sclerospora macrospora</i>
Green ear downy mildew (graminicola downy mildew)	<i>Sclerospora graminicola</i>

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Java downy mildew*	<i>Peronosclerospora maydis</i> = <i>Sclerospora maydis</i>
Philippine downy mildew*	<i>Peronosclerospora philippinensis</i> = <i>Sclerospora philippinensis</i>
Sorghum downy mildew	<i>Peronosclerospora sorghi</i> = <i>Sclerospora sorghi</i>
Spontaneum downy mildew*	<i>Peronosclerospora spontanea</i> = <i>Sclerospora spontanea</i>
Sugarcane downy mildew*	<i>Peronosclerospora sacchari</i> = <i>Sclerospora sacchari</i>
Dry ear rot (cob, kernel and stalk rot)	<i>Nigrospora oryzae</i> (teleomorph: <i>Khuskia oryzae</i>)
Ear rots, minor	<i>Alternaria alternata</i> = <i>A. tenuis</i> , <i>Aspergillus glaucus</i> , <i>A. niger</i> , <i>Aspergillus</i> spp., <i>Botrytis cinerea</i> (teleomorph: <i>Botryotinia fuckeliana</i>), <i>Cunninghamella</i> sp., <i>Curvularia pallescens</i> , <i>Doratomyces stemonitis</i> = <i>Cephalotrichum stemonitis</i> , <i>Fusarium culmorum</i> , <i>Gonatobotrys simplex</i> , <i>Pithomyces maydis</i> , <i>Rhizopus microsporus</i> Tiegh., <i>R. stolonifer</i> = <i>R. nigricans</i> , <i>Scopulariopsis brumptii</i> .
Ergot* (horse's tooth, diente de caballo)	<i>Claviceps gigantea</i> (anamorph: <i>Sphacelia</i> sp.)
Eyespot	<i>Aureobasidium zeae</i> = <i>Kabatiella zeae</i>
Fusarium ear and stalk rot	<i>Fusarium subglutinans</i> = <i>F. moniliforme</i> var. <i>subglutinans</i>

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Fusarium kernel, root and stalk rot, seed rot and seedling blight	<i>Fusarium moniliforme</i> (teleomorph: <i>Gibberella fujikuroi</i>)
Fusarium stalk rot, seedling root rot	<i>Fusarium avenaceum</i> (teleomorph: <i>Gibberella avenacea</i>)
Gibberella ear and stalk rot	<i>Gibberella zeae</i> (anamorph: <i>Fusarium graminearum</i>)
Gray ear rot	<i>Botryosphaeria zeae</i> = <i>Physalospora zeae</i> (anamorph: <i>Macrophoma zeae</i>)
Gray leaf spot (Cercospora leaf spot)	<i>Cercospora sorghi</i> = <i>C. sorghi</i> var. <i>maydis</i> , <i>C. zeae-maydis</i>
Helminthosporium root rot	<i>Exserohilum pedicellatum</i> = <i>Helminthosporium pedicellatum</i> (teleomorph: <i>Setosphaeria pedicellata</i>)
Hormodendrum ear rot (Cladosporium rot)	<i>Cladosporium cladosporioides</i> = <i>Hormodendrum cladosporioides</i> , <i>C. herbarum</i> (teleomorph: <i>Mycosphaerella tassiana</i>)
Hyalothyridium leaf spot*	<i>Hyalothyridium maydis</i>
Late wilt*	<i>Cephalosporium maydis</i>

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Leaf spots, minor	<i>Alternaria alternata</i> , <i>Ascochyta maydis</i> , <i>A. tritici</i> , <i>A. zeicola</i> , <i>Bipolaris victoriae</i> = <i>Helminthosporium victoriae</i> (teleomorph: <i>Cochliobolus victoriae</i>), <i>C. sativus</i> (anamorph: <i>Bipolaris sorokiniana</i> = <i>H.</i> <i>sorokinianum</i> = <i>H. sativum</i>), <i>Epicoccum</i> <i>nigrum</i> , <i>Exserohilum prolatum</i> = <i>Drechslera</i> <i>prolata</i> (teleomorph: <i>Setosphaeria prolata</i>) <i>Graphium penicillioides</i> , <i>Leptosphaeria</i> <i>maydis</i> , <i>Leptothyrium zae</i> , <i>Ophiosphaerella</i> <i>herpotricha</i> , (anamorph: <i>Scolecosporella</i> sp.), <i>Paraphaeosphaeria michotii</i> , <i>Phoma</i> sp., <i>Septoria zae</i> , <i>S. zeicola</i> , <i>S. zeina</i>
Northern corn leaf blight (white blast, crown stalk rot, stripe)	<i>Setosphaeria turcica</i> (anamorph: <i>Exserohilum turcicum</i> = <i>Helminthosporium</i> <i>turcicum</i>)
Northern corn leaf spot, <i>Helminthosporium</i> ear rot (race 1)	<i>Cochliobolus carbonum</i> (anamorph: <i>Bipolaris zeicola</i> = <i>Helminthosporium</i> <i>carbonum</i>)
Penicillium ear rot (blue eye, blue mold)	<i>Penicillium</i> spp., <i>P. chrysogenum</i> , <i>P.</i> <i>expansum</i> , <i>P. oxalicum</i>
<i>Phaeocystostroma</i> stalk rot and root rot	<i>Phaeocystostroma ambiguum</i> , = <i>Phaeocystosporella zae</i>
<i>Phaeosphaeria</i> leaf spot*	<i>Phaeosphaeria maydis</i> = <i>Sphaerulina</i> <i>maydis</i>
<i>Physalospora</i> ear rot (<i>Botryosphaeria</i> ear rot)	<i>Botryosphaeria festucae</i> = <i>Physalospora</i> <i>zeicola</i> (anamorph: <i>Diplodia frumenti</i>)

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Purple leaf sheath	Hemiparasitic bacteria and fungi
Pyrenochaeta stalk rot and root rot	<i>Phoma terrestris</i> = <i>Pyrenochaeta terrestris</i>
Pythium root rot	<i>Pythium</i> spp., <i>P. arrhenomanes</i> , <i>P. graminicola</i>
Pythium stalk rot	<i>Pythium aphanidermatum</i> = <i>P. butleri</i> L.
Red kernel disease (ear mold, leaf and seed rot)	<i>Epicoccum nigrum</i>
Rhizoctonia ear rot (sclerotial rot)	<i>Rhizoctonia zeae</i> (teleomorph: <i>Waitea circinata</i>)
Rhizoctonia root rot and stalk rot	<i>Rhizoctonia solani</i> , <i>Rhizoctonia zeae</i>
Root rots, minor	<i>Alternaria alternata</i> , <i>Cercospora sorghi</i> , <i>Dictyochoeta fertilis</i> , <i>Fusarium acuminatum</i> (teleomorph: <i>Gibberella acuminata</i>), <i>F. equiseti</i> (teleomorph: <i>G. intricans</i>), <i>F. oxysporum</i> , <i>F. pallidoroseum</i> , <i>F. poae</i> , <i>F. roseum</i> , <i>G. cyanogena</i> , (anamorph: <i>F. sulphureum</i>), <i>Microdochium bolleyi</i> , <i>Mucor</i> sp., <i>Periconia circinata</i> , <i>Phytophthora cactorum</i> , <i>P. drechsleri</i> , <i>P. nicotianae</i> var. <i>parasitica</i> , <i>Rhizopus arrhizus</i>
Rostratum leaf spot (Helminthosporium leaf disease, ear and stalk rot)	<i>Setosphaeria rostrata</i> , (anamorph: <i>Exserohilum rostratum</i> = <i>Helminthosporium rostratum</i>)
Rust, common corn	<i>Puccinia sorghi</i>
Rust, southern corn	<i>Puccinia polysora</i>
Rust, tropical corn	<i>Physopella pallescens</i> , <i>P. zeae</i> = <i>Angiopsora zeae</i>
Sclerotium ear rot* (southern blight)	<i>Sclerotium rolfsii</i> Sacc. (teleomorph: <i>Athelia rolfsii</i>)

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Seed rot-seedling blight	<i>Bipolaris sorokiniana</i> , <i>B. zeicola</i> = <i>Helminthosporium carbonum</i> , <i>Diplodia</i> <i>maydis</i> , <i>Exserohilum pedicellatum</i> , <i>Exserohilum turcicum</i> = <i>Helminthosporium</i> <i>turcicum</i> , <i>Fusarium avenaceum</i> , <i>F.</i> <i>culmorum</i> , <i>F. moniliforme</i> , <i>Gibberella zeae</i> (anamorph: <i>F. graminearum</i>), <i>Macrophomina phaseolina</i> , <i>Penicillium</i> spp., <i>Phomopsis</i> sp., <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>R. zeae</i> , <i>Sclerotium</i> <i>rolfsii</i> , <i>Spicaria</i> sp.
Selenophoma leaf spot*	<i>Selenophoma</i> sp.
Sheath rot	<i>Gaeumannomyces graminis</i>
Shuck rot	<i>Myrothecium gramineum</i>
Silage mold	<i>Monascus purpureus</i> , <i>M. ruber</i>
Smut, common	<i>Ustilago zeae</i> = <i>U. maydis</i>)
Smut, false	<i>Ustilaginoidea virens</i>
Smut, head	<i>Sphacelotheca reiliana</i> = <i>Sporisorium holci-</i> <i>sorghii</i>
Southern corn leaf blight and stalk rot	<i>Cochliobolus heterostrophus</i> (anamorph: <i>Bipolaris maydis</i> = <i>Helminthosporium</i> <i>maydis</i>)
Southern leaf spot	<i>Stenocarpella macrospora</i> = <i>Diplodia</i> <i>macrospora</i>

Table 5 - Continued
Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Stalk rots, minor	<i>Cercospora sorghi</i> , <i>Fusarium episphaeria</i> , <i>F. merismoides</i> , <i>F. oxysporum</i> Schlechtend, <i>F. poae</i> , <i>F. roseum</i> , <i>F. solani</i> (teleomorph: <i>Nectria haematococca</i>), <i>F. tricinatum</i> , <i>Mariannaea elegans</i> , <i>Mucor</i> sp., <i>Rhopoglyphus zeae</i> , <i>Spicaria</i> sp.
Storage rots	<i>Aspergillus</i> spp., <i>Penicillium</i> spp. and other fungi
Tar spot*	<i>Phyllachora maydis</i>
Trichoderma ear rot and root rot	<i>Trichoderma viride</i> = <i>T. lignorum</i> teleomorph: <i>Hypocrea</i> sp.
White ear rot, root and stalk rot	<i>Stenocarpella maydis</i> = <i>Diplodia zeae</i>
Yellow leaf blight	<i>Ascochyta ischaemi</i> , <i>Phyllosticta maydis</i> (teleomorph: <i>Mycosphaerella zeae-maydis</i>)
Zonate leaf spot	<i>Gloeocercospora sorghi</i>

*Not known to occur naturally on corn in the United States.

- 5 Plant parasitic nematodes are a cause of disease in many plants, including maize. It is proposed that it would be possible to make the corn plant resistant to these organisms through the expression of novel genes. It is anticipated that control of nematode infestations would be accomplished by altering the ability of the nematode to recognize or attach to a host plant and/or enabling the plant to produce nematocidal compounds, including but not limited to proteins.
- 10 Examples of nematode-associated plant diseases, for which one could introduce resistance to in a transgenic plant in accordance with the invention are given below, in Table 6.

Table 6
Parasitic Nematodes

DISEASE	PATHOGEN
Awl	<i>Dolichodorus</i> spp., <i>D. heterocephalus</i>
Bulb and stem (Europe)	<i>Ditylenchus dipsaci</i>
Burrowing	<i>Radopholus similis</i>
Cyst	<i>Heterodera avenae</i> , <i>H. zae</i> , <i>Punctodera</i> <i>chalcoensis</i>
Dagger	<i>Xiphinema</i> spp., <i>X. americanum</i> , <i>X.</i> <i>mediterraneum</i>
False root-knot	<i>Nacobbus dorsalis</i>
Lance, Columbia	<i>Hoplolaimus columbus</i>
Lance	<i>Hoplolaimus</i> spp., <i>H. galeatus</i>
Lesion	<i>Pratylenchus</i> spp., <i>P. brachyurus</i> , <i>P.</i> <i>crenatus</i> , <i>P. hexincisus</i> , <i>P. neglectus</i> , <i>P.</i> <i>penetrans</i> , <i>P. scribneri</i> , <i>P. thornei</i> , <i>P. zae</i>
Needle	<i>Longidorus</i> spp., <i>L. breviannulatus</i>
Ring	<i>Criconemella</i> spp., <i>C. ornata</i>
Root-knot	<i>Meloidogyne</i> spp., <i>M. chitwoodi</i> , <i>M.</i> <i>incognita</i> , <i>M. javanica</i>
Spiral	<i>Helicotylenchus</i> spp.
Sting	<i>Belonolaimus</i> spp., <i>B. longicaudatus</i>
Stubby-root	<i>Paratrichodorus</i> spp., <i>P. christiei</i> , <i>P. minor</i> , <i>Quinisulcius acutus</i> , <i>Trichodorus</i> spp.
Stunt	<i>Tylenchorhynchus dubius</i>

(v) *Mycotoxin Reduction/Elimination*

- 5 Production of mycotoxins, including aflatoxin and fumonisin, by fungi associated with monocotyledonous plants such as maize is a significant factor in rendering the grain not useful. These fungal organisms do not cause disease symptoms and/or interfere with the growth of the plant, but they produce chemicals (mycotoxins) that are toxic to animals. It is contemplated that inhibition of the growth of these fungi would reduce the synthesis of these toxic substances and

therefore reduce grain losses due to mycotoxin contamination. It also is proposed that it may be possible to introduce novel genes into monocotyledonous plants such as maize that would inhibit synthesis of the mycotoxin. Further, it is contemplated that expression of a novel gene which encodes an enzyme capable of rendering the mycotoxin nontoxic would be useful in order to achieve reduced mycotoxin contamination of grain. The result of any of the above mechanisms would be a reduced presence of mycotoxins on grain.

(vi) *Grain Composition or Quality*

Genes may be introduced into monocotyledonous plants, particularly commercially important cereals such as maize, to improve the grain for which the cereal is primarily grown. A wide range of novel transgenic plants produced in this manner may be envisioned depending on the particular end use of the grain.

The largest use of maize grain is for feed or food. Introduction of genes that alter the composition of the grain may greatly enhance the feed or food value. The primary components of maize grain are starch, protein, and oil. Each of these primary components of maize grain may be improved by altering its level or composition. Several examples may be mentioned for illustrative purposes, but in no way provide an exhaustive list of possibilities.

The protein of cereal grains including maize is suboptimal for feed and food purposes especially when fed to pigs, poultry, and humans. The protein is deficient in several amino acids that are essential in the diet of these species, requiring the addition of supplements to the grain. Limiting essential amino acids may include lysine, methionine, tryptophan, threonine, valine, arginine, and histidine. Some amino acids become limiting only after corn is supplemented with other inputs for feed formulations. For example, when corn is supplemented with soybean meal to meet lysine requirements methionine becomes limiting. The levels of these essential amino acids in seeds and grain may be elevated by mechanisms which include, but are not limited to, the introduction of genes to increase the biosynthesis of the amino acids, decrease the degradation of the amino acids, increase the storage of the amino acids in proteins, or increase transport of the amino acids to the seeds or grain.

One mechanism for increasing the biosynthesis of the amino acids is to introduce genes that deregulate the amino acid biosynthetic pathways such that the plant can no longer adequately control the levels that are produced. This may be done by deregulating or bypassing steps in the amino acid biosynthetic pathway which are normally regulated by levels of the amino acid end product of the pathway. Examples include the introduction of genes that encode deregulated versions of the enzymes aspartokinase or dihydrodipicolinic acid (DHDP)-synthase for increasing lysine and threonine production, and anthranilate synthase for increasing tryptophan production. Reduction of the catabolism of the amino acids may be accomplished by introduction of DNA sequences that reduce or eliminate the expression of genes encoding enzymes that catalyze steps in the catabolic pathways such as the enzyme lysine-ketoglutarate reductase. It is anticipated that it may be desirable to target expression of genes relating to amino acid biosynthesis to the endosperm or embryo of the seed. More preferably, the gene will be targeted to the embryo. It will also be preferable for genes encoding proteins involved in amino acid biosynthesis to target the protein to a plastid using a plastid transit peptide sequence.

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The protein composition of the grain may be altered to improve the balance of amino acids in a variety of ways including elevating expression of native proteins, decreasing expression of those with poor composition, changing the composition of native proteins, or introducing genes encoding entirely new proteins possessing superior composition. Examples may include the introduction of DNA that decreases the expression of members of the zein family of storage proteins. This DNA may encode ribozymes or antisense sequences directed to impairing expression of zein proteins or expression of regulators of zein expression such as the opaque-2 gene product. It also is proposed that the protein composition of the grain may be modified through the phenomenon of co-suppression, *i.e.*, inhibition of expression of an endogenous gene through the expression of an identical structural gene or gene fragment introduced through transformation (Goring *et al.*, 1991). Additionally, the introduced DNA may encode enzymes which degrade zeins. The decreases in zein expression that are achieved may be accompanied by increases in proteins with more desirable amino acid composition or increases in other major seed constituents such as starch. Alternatively, a chimeric gene may be introduced that comprises a coding sequence for a native protein of adequate amino acid composition such as for one of the globulin proteins or 10 kD delta zein or 20 kD delta zein or 27 kD gamma zein of maize and a promoter or other regulatory sequence designed to elevate expression of said protein. The

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coding sequence of said gene may include additional or replacement codons for essential amino acids. Further, a coding sequence obtained from another species, or, a partially or completely synthetic sequence encoding a completely unique peptide sequence designed to enhance the amino acid composition of the seed may be employed. It is anticipated that it may be preferable to target expression of these transgenes encoding proteins with superior composition to the endosperm of the seed.

The introduction of genes that alter the oil content of the grain may be of value. Increases in oil content may result in increases in metabolizable-energy-content and density of the seeds for use in feed and food. The introduced genes may encode enzymes that remove or reduce rate-limitations or regulated steps in fatty acid or lipid biosynthesis. Such genes may include, but are not limited to, those that encode acetyl-CoA carboxylase, ACP-acyltransferase, β -ketoacyl-ACP synthase, plus other well known fatty acid biosynthetic activities. Other possibilities are genes that encode proteins that do not possess enzymatic activity such as acyl carrier protein. Genes may be introduced that alter the balance of fatty acids present in the oil providing a more healthful or nutritive feedstuff. The introduced DNA also may encode sequences that block expression of enzymes involved in fatty acid biosynthesis, altering the proportions of fatty acids present in the grain such as described below. Some other examples of genes specifically contemplated by the inventors for use in creating transgenic plants with altered oil composition traits include 2-acetyltransferase, oleosin, pyruvate dehydrogenase complex, acetyl CoA synthetase, ATP citrate lyase, ADP-glucose pyrophosphorylase and genes of the carnitine-CoA-acetyl-CoA shuttles. It is anticipated that expression of genes related to oil biosynthesis will be targeted to the plastid, using a plastid transit peptide sequence and preferably expressed in the seed embryo.

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Genes may be introduced that enhance the nutritive value of the starch component of the grain, for example by increasing the degree of branching, resulting in improved utilization of the starch in cows by delaying its metabolism. It is anticipated that expression of genes related to starch biosynthesis will preferably be targeted to the endosperm of the seed.

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Besides affecting the major constituents of the grain, genes may be introduced that affect a variety of other nutritive, processing, or other quality aspects of the grain as used for feed or

food. For example, pigmentation of the grain may be increased or decreased. Enhancement and stability of yellow pigmentation is desirable in some animal feeds and may be achieved by introduction of genes that result in enhanced production of xanthophylls and carotenes by eliminating rate-limiting steps in their production. Such genes may encode altered forms of the enzymes phytoene synthase, phytoene desaturase, or lycopene synthase. Alternatively, unpigmented white corn is desirable for production of many food products and may be produced by the introduction of DNA which blocks or eliminates steps in pigment production pathways.

Most of the phosphorous content of the grain is in the form of phytate, a form of phosphate storage that is not metabolized by monogastric animals. Therefore, in order to increase the availability of seed phosphate, it is anticipated that one will desire to decrease the amount of phytate in seed and increase the amount of free phosphorous. It is anticipated that one can decrease the expression or activity of one of the enzymes involved in the synthesis of phytate. For example, suppression of expression of the gene encoding inositol phosphate synthetase (INOPS) may lead to an overall reduction in phytate accumulation. It is anticipated that antisense or sense suppression of gene expression may be used. Alternatively, one may express a gene in corn seed which will be activated, *e.g.*, by pH, in the gastric system of a monogastric animal and will release phosphate from phytate, *e.g.*, phytase.

Feed or food comprising primarily maize or other cereal grains possesses insufficient quantities of vitamins and must be supplemented to provide adequate nutritive value. Introduction of genes that enhance vitamin biosynthesis in seeds may be envisioned including, for example, vitamins A, E, B₁₂, choline, and the like. Maize grain also does not possess sufficient mineral content for optimal nutritive value. Genes that affect the accumulation or availability of compounds containing phosphorus, sulfur, calcium, manganese, zinc, and iron among others would be valuable. An example may be the introduction of a gene that reduced phytic acid production or encoded the enzyme phytase which enhances phytic acid breakdown. These genes would increase levels of available phosphate in the diet, reducing the need for supplementation with mineral phosphate.

Numerous other examples of improvement of maize or other cereals for feed and food purposes might be described. The improvements may not even necessarily involve the grain, but

may, for example, improve the value of the corn for silage. Introduction of DNA to accomplish this might include sequences that alter lignin production such as those that result in the "brown midrib" phenotype associated with superior feed value for cattle.

5 In addition to direct improvements in feed or food value, genes also may be introduced which improve the processing of corn and improve the value of the products resulting from the processing. The primary method of processing corn is via wetmilling. Maize may be improved through the expression of novel genes that increase the efficiency and reduce the cost of processing such as by decreasing steeping time.

10 Improving the value of wetmilling products may include altering the quantity or quality of starch, oil, corn gluten meal, or the components of corn gluten feed. Elevation of starch may be achieved through the identification and elimination of rate limiting steps in starch biosynthesis or by decreasing levels of the other components of the grain resulting in proportional increases in
15 starch. An example of the former may be the introduction of genes encoding ADP-glucose pyrophosphorylase enzymes with altered regulatory activity or which are expressed at higher level. Examples of the latter may include selective inhibitors of, for example, protein or oil biosynthesis expressed during later stages of kernel development.

20 The properties of starch may be beneficially altered by changing the ratio of amylose to amylopectin, the size of the starch molecules, or their branching pattern. Through these changes a broad range of properties may be modified which include, but are not limited to, changes in gelatinization temperature, heat of gelatinization, clarity of films and pastes, rheological properties, and the like. To accomplish these changes in properties, genes that encode granule-
25 bound or soluble starch synthase activity or branching enzyme activity may be introduced alone or combination. DNA such as antisense constructs also may be used to decrease levels of endogenous activity of these enzymes. The introduced genes or constructs may possess regulatory sequences that time their expression to specific intervals in starch biosynthesis and starch granule development. Furthermore, it may be worthwhile to introduce and express genes
30 that result in the *in vivo* derivatization, or other modification, of the glucose moieties of the starch molecule. The covalent attachment of any molecule may be envisioned, limited only by the existence of enzymes that catalyze the derivatizations and the accessibility of appropriate

substrates in the starch granule. Examples of important derivations may include the addition of functional groups such as amines, carboxyls, or phosphate groups which provide sites for subsequent *in vitro* derivatizations or affect starch properties through the introduction of ionic charges. Examples of other modifications may include direct changes of the glucose units such as
5 loss of hydroxyl groups or their oxidation to aldehyde or carboxyl groups.

Oil is another product of wetmilling of corn, the value of which may be improved by introduction and expression of genes. The quantity of oil that can be extracted by wetmilling may be elevated by approaches as described for feed and food above. Oil properties also may be
10 altered to improve its performance in the production and use of cooking oil, shortenings, lubricants or other oil-derived products or improvement of its health attributes when used in the food-related applications. Novel fatty acids also may be synthesized which upon extraction can serve as starting materials for chemical syntheses. The changes in oil properties may be achieved by altering the type, level, or lipid arrangement of the fatty acids present in the oil. This in turn
15 may be accomplished by the addition of genes that encode enzymes that catalyze the synthesis of novel fatty acids and the lipids possessing them or by increasing levels of native fatty acids while possibly reducing levels of precursors. Alternatively, DNA sequences may be introduced which slow or block steps in fatty acid biosynthesis resulting in the increase in precursor fatty acid intermediates. Genes that might be added include desaturases, epoxidases, hydratases,
20 dehydratases, and other enzymes that catalyze reactions involving fatty acid intermediates. Representative examples of catalytic steps that might be blocked include the desaturations from stearic to oleic acid and oleic to linolenic acid resulting in the respective accumulations of stearic and oleic acids. Another example is the blockage of elongation steps resulting in the accumulation of C₈ to C₁₂ saturated fatty acids.

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Improvements in the other major corn wetmilling products, corn gluten meal and corn gluten feed, also may be achieved by the introduction of genes to obtain novel corn plants. Representative possibilities include but are not limited to those described above for improvement of food and feed value.

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In addition, it may further be considered that the corn plant be used for the production or manufacturing of useful biological compounds that were either not produced at all, or not

produced at the same level, in the corn plant previously. The novel corn plants producing these compounds are made possible by the introduction and expression of genes by corn transformation methods. The vast array of possibilities include but are not limited to any biological compound which is presently produced by any organism such as proteins, nucleic acids, primary and intermediary metabolites, carbohydrate polymers, *etc.* The compounds may be produced by the plant, extracted upon harvest and/or processing, and used for any presently recognized useful purpose such as pharmaceuticals, fragrances, and industrial enzymes to name a few. For example, expression of interferon in maize that is consumed by an animal may lead to increased resistance to viral infections on the part of the animal, *e.g.*, feeding of γ -interferon to chickens in corn may reduce the occurrence of chicken viral infection.

Further possibilities to exemplify the range of grain traits or properties potentially encoded by introduced genes in transgenic plants include grain with less breakage susceptibility for export purposes or larger grit size when processed by dry milling through introduction of genes that enhance γ -zein synthesis, popcorn with improved popping quality and expansion volume through genes that increase pericarp thickness, corn with whiter grain for food uses though introduction of genes that effectively block expression of enzymes involved in pigment production pathways, and improved quality of alcoholic beverages or sweet corn through introduction of genes which affect flavor such as the shrunken 1 gene (encoding sucrose synthase) or shrunken 2 gene (encoding ADPG pyrophosphorylase) for sweet corn.

(vii) *Plant Agronomic Characteristics*

Two of the factors determining where crop plants can be grown are the average daily temperature during the growing season and the length of time between frosts. Within the areas where it is possible to grow a particular crop, there are varying limitations on the maximal time it is allowed to grow to maturity and be harvested. For example, maize to be grown in a particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, corn of varying maturities is developed for different growing locations. Apart from the need to dry down sufficiently to permit harvest, it is desirable to have maximal drying take place in the field to minimize the amount of energy required for additional drying post-harvest. Also, the more readily the grain can dry down, the more time there is available for growth and kernel fill. It is considered that

genes that influence maturity and/or dry down can be identified and introduced into corn or other plant lines using transformation techniques to create new varieties adapted to different growing locations or the same growing location, but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially useful, e.g., the liguleless and rough sheath genes that have been identified in corn.

It further is contemplated that expression of a gene encoding a hemoglobin gene or other oxygen binding protein may enhance the growth and yield of a plant through an increase in oxygen utilization and metabolic efficiency. For example, expression of a gene encoding an oxygen binding protein may enhance the ability of a plant to resist flooding, stress, increase seedling vigor, or increase yield. Examples of oxygen binding proteins are the hemoglobin gene isolated from *Vitroscilla* (Khosla and Bailey, 1988; GenBank Accession No. X13516) and expressed in tobacco (Holmberg *et al.*, 1997), two hemoglobin encoding genes from rice (Arrendondo-Peter *et al.*, 1997; GenBank Accession Nos. U76030 and U76031), and a hemoglobin encoding gene from barley (Guy *et al.*, 1997; GenBank Accession No. U94968).

It is contemplated that genes may be introduced into plants that would improve standability and other plant growth characteristics. Expression of novel genes in maize which confer stronger stalks, improved root systems, or prevent or reduce ear droppage would be of great value to the farmer. It is proposed that introduction and expression of genes that increase the total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In addition, the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further increase gains in productivity. It is contemplated that expression of a phytochrome gene in corn may be advantageous. Expression of such a gene may reduce apical dominance, confer semidwarfism on a plant, and increase shade tolerance (U.S. Patent No. 5,268,526). Such approaches would allow for increased plant populations in the field.

Delay of late season vegetative senescence would increase the flow of assimilate into the grain and thus increase yield. It is proposed that overexpression of genes within corn that are associated with "stay green" or the expression of any gene that delays senescence would be advantageous. For example, a nonyellowing mutant has been identified in *Festuca pratensis*

(Davies *et al.*, 1990). Expression of this gene as well as others may prevent premature breakdown of chlorophyll and thus maintain canopy function.

(viii) *Nutrient Utilization*

5 The ability to utilize available nutrients may be a limiting factor in growth of monocotyledonous plants such as maize. It is proposed that it would be possible to alter nutrient uptake, tolerate pH extremes, mobilization through the plant, storage pools, and availability for metabolic activities by the introduction of novel genes. These modifications would allow a plant such as maize to more efficiently utilize available nutrients. It is contemplated that an increase in
10 the activity of, for example, an enzyme that is normally present in the plant and involved in nutrient utilization would increase the availability of a nutrient. An example of such an enzyme would be phytase. It is further contemplated that enhanced nitrogen utilization by a plant is desirable. Expression of a glutamate dehydrogenase gene in corn, *e.g.*, *E. coli* *gdhA* genes, may lead to increased fixation of nitrogen in organic compounds. Furthermore, expression of *gdhA* in
15 corn may lead to enhanced resistance to the herbicide glufosinate by incorporation of excess ammonia into glutamate, thereby detoxifying the ammonia. It also is contemplated that expression of a novel gene may make a nutrient source available that was previously not accessible, *e.g.*, an enzyme that releases a component of nutrient value from a more complex molecule, perhaps a macromolecule.

20 (ix) *Male Sterility*

Male sterility is useful in the production of hybrid seed. It is proposed that male sterility may be produced through expression of novel genes. For example, it has been shown that expression of genes that encode proteins that interfere with development of the male
25 inflorescence and/or gametophyte result in male sterility. Chimeric ribonuclease genes that express in the anthers of transgenic tobacco and oilseed rape have been demonstrated to lead to male sterility (Mariani *et al.*, 1990).

A number of mutations were discovered in maize that confer cytoplasmic male sterility.
30 One mutation in particular, referred to as T cytoplasm, also correlates with sensitivity to Southern corn leaf blight. A DNA sequence, designated TURF-13 (Levings, 1990), was identified that correlates with T cytoplasm. It is proposed that it would be possible through the

introduction of TURF-13 via transformation, to separate male sterility from disease sensitivity. As it is necessary to be able to restore male fertility for breeding purposes and for grain production, it is proposed that genes encoding restoration of male fertility also may be introduced. It is anticipated that male sterility may be achieved by application of a chemical compound which inhibits development of pollen. For example, a herbicide may be applied to a plant wherein the vegetative and female tissues of the plant are resistant to the herbicide and the male reproductive tissues are sensitive to the herbicide. U.S. Patent Application 08/927,368 describes the use of glyphosate to render corn plants male sterile.

(x) *Negative Selectable Markers*

Introduction of genes encoding traits that can be selected against may be useful for eliminating undesirable linked genes. It is contemplated that when two or more genes are introduced together by cotransformation that the genes will be linked together on the host chromosome. For example, a gene encoding a *Bt* gene that confers insect resistance on the plant may be introduced into a plant together with a *bar* gene that is useful as a selectable marker and confers resistance to the herbicide Liberty® on the plant. However, it may not be desirable to have an insect resistant plant that also is resistant to the herbicide Liberty®. It is proposed that one also could introduce an antisense *bar* gene that is expressed in those tissues where one does not want expression of the *bar* gene, e.g., in whole plant parts. Hence, although the *bar* gene is expressed and is useful as a selectable marker, it is not useful to confer herbicide resistance on the whole plant. The *bar* antisense gene is a negative selectable marker.

It also is contemplated that negative selection is necessary in order to screen a population of transformants for rare homologous recombinants generated through gene targeting. For example, a homologous recombinant may be identified through the inactivation of a gene that was previously expressed in that cell. The antisense gene to neomycin phosphotransferase II (NPT II) has been investigated as a negative selectable marker in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Xiang, and Guerra, 1993). In this example, both sense and antisense NPT II genes are introduced into a plant through transformation and the resultant plants are sensitive to the antibiotic kanamycin. An introduced gene that integrates into the host cell chromosome at the site of the antisense NPT II gene, and inactivates the antisense gene, will make the plant resistant to kanamycin and other aminoglycoside antibiotics. Therefore, rare, site-specific

recombinants may be identified by screening for antibiotic resistance. Similarly, any gene, native to the plant or introduced through transformation, that when inactivated confers resistance to a compound, may be useful as a negative selectable marker.

5 It is contemplated that negative selectable markers also may be useful in other ways. One application is to construct transgenic lines in which one could select for transposition to unlinked sites. In the process of tagging it is most common for the transposable element to move to a genetically linked site on the same chromosome. A selectable marker for recovery of rare plants in which transposition has occurred to an unlinked locus would be useful. For example, the
10 enzyme cytosine deaminase may be useful for this purpose (Stouggard, 1993). In the presence of this enzyme the compound 5-fluorocytosine is converted to 5-fluorouracil which is toxic to plant and animal cells. If a transposable element is linked to the gene for the enzyme cytosine deaminase, one may select for transposition to unlinked sites by selecting for transposition events in which the resultant plant is now resistant to 5-fluorocytosine. The parental plants and plants
15 containing transpositions to linked sites will remain sensitive to 5-fluorocytosine. Resistance to 5-fluorocytosine is due to loss of the cytosine deaminase gene through genetic segregation of the transposable element and the cytosine deaminase gene. Other genes that encode proteins that render the plant sensitive to a certain compound will also be useful in this context. For example, T-DNA gene 2 from *Agrobacterium tumefaciens* encodes a protein that catalyzes the conversion
20 of α -naphthalene acetamide (NAM) to α -naphthalene acetic acid (NAA) renders plant cells sensitive to high concentrations of NAM (Depicker *et al.*, 1988).

 It also is contemplated that negative selectable markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such
25 as Ac, Master Mu, or En/Spn with a negative selectable marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. It is proposed that this would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as Ds, but stable integration of the autonomous element is not desired. The presence of the
30 autonomous element may not be desired in order to stabilize the defective element, *i.e.*, prevent it from further transposing. However, it is proposed that if stable integration of an autonomous

transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.

(xi) *Non-Protein-Expressing Sequences*

5 DNA may be introduced into plants for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes. However, as detailed below, DNA need not be expressed to effect the phenotype of a plant.

10 1. Antisense RNA

Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product
15 may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of the reaction which include
20 any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited above are provided only by way of example and do not represent the full range of applications.

25 2. Ribozymes

Genes also may be constructed or isolated, which when transcribed, produce RNA enzymes (ribozymes) which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNAs can result in the
30 reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including, but not limited to, the polypeptides cited above.

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often
5 cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific
10 cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes.

Several different ribozyme motifs have been described with RNA cleavage activity
15 (Symons, 1992). Examples include sequences from the Group I self splicing introns including Tobacco Ringspot Virus (Prody *et al.*, 1986), Avocado Sunblotch Viroid (Palukaitis *et al.*, 1979; Symons, 1981), and Lucerne Transient Streak Virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozyme based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity
20 (Yuan *et al.*, 1992, Yuan and Altman, 1994, U. S. Patents 5,168,053 and 5,624,824), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and Hepatitis Delta virus based ribozymes (U. S. Patent 5,625,047). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach,
25 1988, Symons, 1992, Chowrira *et al.*, 1994; Thompson *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above.

Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozyme, the cleavage site is a dinucleotide sequence on the target RNA is a uracil (U) followed by either an adenine, cytosine or uracil (A,C or U) (Perriman *et al.*, 1992; Thompson *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible.

Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in down regulating a given gene is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

3. Induction of Gene Silencing

It also is possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by the mechanism of co-suppression. It has been demonstrated in tobacco, tomato, and petunia (Goring *et al.*, 1991; Smith *et al.*, 1990; Napoli *et al.*, 1990; van der Krol *et al.*, 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

4. Non-RNA-Expressing Sequences

DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be inserted into a gene to cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, *e.g.*, using the

introduced DNA sequence as a PCR primer together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta *et al.*, 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired, may be isolated, cloned and manipulated as desired. The utility of DNA elements introduced into an organism for purposes of gene tagging
5 is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, *i.e.*, transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

It is contemplated that unexpressed DNA sequences, including novel synthetic sequences,
10 could be introduced into cells as proprietary "labels" of those cells and plants and seeds thereof. It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that
15 labeled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief, 1989), which can be positioned around
20 an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependent effects upon incorporation into the plant genome (Stief *et al.*, 1989; Phi-Van *et al.*, 1990).

III. Assays of Transgene Expression

25 Assays may be employed with the instant invention for determination of the relative efficiency of transgene expression. For example, assays may be used to determine the efficacy of deletion mutants of the actin 2 promoter for expression of exogenous genes. Alternatively, assays could be used to determine the efficacy of the actin 2 intron in enhancing gene expression when used in conjunction with various different promoters and exogenous genes.

30

For plants, expression assays may comprise a system utilizing embryogenic or non-embryogenic cells, or alternatively, whole plants. An advantage of using cellular assays is that

regeneration of large numbers of plants is not required. However, the systems are limited in that promoter activity in the non-regenerated cells may not directly correlate with expression in a plant. Additionally, assays of tissue or developmental specific promoters are generally not feasible.

5

The biological sample to be assayed may comprise nucleic acids isolated from the cells of any plant material according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment of the invention, the
10 RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by
15 visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product *via* chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

20 Following detection, one may compare the results seen in a given plant with a statistically significant reference group of non-transformed control plants. Typically, the non-transformed control plants will be of a genetic background similar to the transformed plants. In this way, it is possible to detect differences in the amount or kind of protein detected in various transformed plants. Alternatively, clonal cultures of cells, for example, callus or an immature embryo, may be
25 compared to other cells samples.

As indicated, a variety of different assays are contemplated in the screening of cells or plants of the current invention and associated promoters. These techniques may in cases be used to detect for both the presence and expression of the particular genes as well as rearrangements
30 that may have occurred in the gene construct. The techniques include but are not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, pulsed field gel electrophoresis (PFGE) analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA),

RNAse protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCRTM-SSCP.

(i) *Quantitation of Gene Expression with Relative Quantitative RT- PCRTM*

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCRTM (RT-PCRTM) can be used to determine the relative concentrations of specific mRNA species isolated from plants. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed. In this way, a promoters expression profile can be rapidly identified.

In PCRTM, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCRTM amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCRTM reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCRTM products and the relative mRNA abundances is only true in the linear range of the PCRTM reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR™ for a collection of RNA populations is that the concentrations of the amplified PCR™ products must be sampled when the PCR™ reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR™ study to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR™ study is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

Most protocols for competitive PCR™ utilize internal PCR™ standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR™ amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR™ assay for plant tissue. The problems inherent in plant tissue samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of larger size than the target). Both of these problems are overcome if the RT-PCR™ is performed as a relative quantitative RT-PCR™ with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal

standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR™ assay with an external standard protocol. These assays sample the PCR™ products in the linear portion of their amplification curves. The number of PCR™ cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR™ assays can be superior to those derived from the relative quantitative RT-PCR™ assay with an internal standard.

One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR™ product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR™ product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

(ii) *Marker Gene Expression*

Marker genes represent an efficient means for assaying the expression of transgenes. Using, for example, a selectable marker gene, one could quantitatively determine the resistance conferred upon a plant or plant cell by a construct comprising the selectable marker gene operably linked to the promoter to be assayed, e.g., an actin 2 promoter. Alternatively, various plant parts could be exposed to a selective agent and the relative resistance provided in these parts quantified, thereby providing an estimate of the tissue specific expression of the promoter.

Screenable markers constitute another efficient means for quantifying the expression of a given transgene. Potentially any screenable marker could be expressed and the marker gene

product quantified, thereby providing an estimate of the efficiency with which the promoter directs expression of the transgene. Quantification can readily be carried out using either visual means, or, for example, a photon counting device.

A preferred screenable marker gene assay for use with the current invention constitutes the use of the screenable marker gene β -glucuronidase (GUS). Detection of GUS activity can be performed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as the substrate for the GUS enzyme, yielding a blue precipitate inside of cells containing GUS activity. This assay has been described in detail (Jefferson, 1987). The blue coloration can then be visually scored, and estimates of expression efficiency thereby provided. GUS activity also can be determined by immunoblot analysis or a fluorometric GUS specific activity assay (Jefferson, 1987).

(iii) Purification and Assays of Proteins

One means for determining the efficiency with which a particular transgene is expressed is to purify and quantify a polypeptide expressed by the transgene. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography, polyacrylamide gel electrophoresis, and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification

steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted

from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to
5 molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one
10 of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate
15 containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the
20 purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in
25 obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide
30 relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in

accord with the present invention is discussed below.

IV. Methods for Mutagenizing Regulatory Elements

It is specifically contemplated by the inventors that one could mutagenize the actin 2
5 promoter or actin 2 intron of the invention to potentially improve the utility of the elements for
the expression of transgenes in plants. The mutagenesis of these elements could be carried out at
random and the mutagenized actin 2 sequences screened for utility in a trial-by-error procedure.
Alternatively, particular sequences which provide the actin 2 promoter with desirable expression
characteristics, or the actin 2 intron with expression enhancement activity, could be identified and
10 these or similar sequences introduced into the sequences via mutation. It is further contemplated
that one could mutagenize these sequences in order to enhance their utility in expressing
transgenes in a particular species, for example, in maize.

The means for mutagenizing a DNA segment encoding an actin 2 sequence of the current
15 invention are well-known to those of skill in the art. As indicated, modifications to promoter or
intron regions may be made by random, or site-specific mutagenesis procedures. The promoter
and intron regions may be modified by altering their structure through the addition or deletion of
one or more nucleotides from the sequence which encodes the corresponding un-modified
sequences.

20

Mutagenesis may be performed in accordance with any of the techniques known in the
art, such as, and not limited to, synthesizing an oligonucleotide having one or more mutations
within the sequence of a particular regulatory region. In particular, site-specific mutagenesis is a
technique useful in the preparation of promoter mutants, through specific mutagenesis of the
25 underlying DNA. The technique further provides a ready ability to prepare and test sequence
variants, for example, incorporating one or more of the foregoing considerations, by introducing
one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the
production of mutants through the use of specific oligonucleotide sequences which encode the
DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to
30 provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on
both sides of the deletion junction being traversed. Typically, a primer of about 17 to about 75

nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids also are routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the actin 2 intron or actin 2 promoter. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and cells are selected which include recombinant vectors bearing the mutated sequence arrangement. Vector DNA can then be isolated from these cells and used for plant transformation. A genetic selection scheme was devised by Kunkel *et al.* (1987) to enrich for clones incorporating mutagenic oligonucleotides. Alternatively, the use of PCRTM with commercially available thermostable enzymes such as *Taq* polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCRTM-mediated mutagenesis procedures of Tomic *et al.* (1990) and Upender *et al.* (1995) provide two examples of such protocols. A PCRTM employing a thermostable ligase in addition to a thermostable polymerase also may be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector. The mutagenesis procedure described by Michael (1994) provides an example of one such protocol.

The preparation of sequence variants of the selected promoter-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained. For example, recombinant vectors encoding the desired promoter sequence
5 may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the
10 concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" also is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template-dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of
15 the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson and Ramstad, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U.S. Pat. No. 4,237,224, specifically
20 incorporated herein by reference in its entirety. A number of template dependent processes are available to amplify the target sequences of interest present in a sample, such methods being well known in the art and specifically disclosed herein below.

Where a clone comprising a promoter has been isolated in accordance with the instant
25 invention, it is contemplated that one may wish to delimit the essential promoter regions within the clone. One efficient, targeted means for preparing mutagenizing promoters relies upon the identification of putative regulatory elements within the promoter sequence. This can be initiated by comparison with promoter sequences known to be expressed in similar tissue-specific or developmentally unique manner. Sequences which are shared among promoters with similar
30 expression patterns are likely candidates for the binding of transcription factors and are thus likely elements which confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by functional

analysis of each deletion construct by assay of a reporter gene which is functionally attached to each construct. As such, once a starting promoter sequence is provided, any of a number of different deletion mutants of the starting promoter could be readily prepared.

5 As indicated above, deletion mutants, deletion mutants of the actin 2 promoter or actin 2 intron of the invention also could be randomly prepared and then assayed. With this strategy, a series of constructs are prepared, each containing a different portion of the clone (a subclone), and these constructs are then screened for activity. A suitable means for screening for activity is to attach a deleted promoter or intron construct which contains a deleted segment to a selectable
10 or screenable marker, and to isolate only those cells expressing the marker gene. In this way, a number of different, deleted promoter constructs are identified which still retain the desired, or even enhanced, activity. The smallest segment which is required for activity is thereby identified through comparison of the selected constructs. This segment may then be used for the construction of vectors for the expression of exogenous genes.

15

V. Methods for Plant Transformation

There are many methods for transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as
20 by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaepler *et al.*, 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No.
25 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of
30 techniques such as these, maize cells as well as those of virtually any other plant species may be stably transformed, and these cells developed into transgenic plants. In certain embodiments,

acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

(i) *Electroporation*

5 Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Patent No. 5,384,253, incorporated herein by reference in its entirety) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells
10 are made more susceptible to transformation by mechanical wounding.

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or
15 mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, 1995; D'Halluin *et al.*, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates,
20 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazzeri, 1995), sorghum (Battraw *et al.*, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and
25 tomato (Tsukada, 1989).

(ii) *Microprojectile Bombardment*

A preferred method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,489,520; U.S.

Patent No. 5,538,880; U.S. Patent No. 5,610, 042; and PCT Application WO 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128; U.S. Patent No. 5,538,880; U.S. Patent 5,489,520), barley (Ritala *et al.*, 1994; Hensgens *et al.*, 1993), wheat (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety), rice (Hensgens *et al.*, 1993), oat (Torbet *et al.*, 1995; Torbet *et al.*, 1998), rye (Hensgens *et al.*, 1993), sugarcane (Bower *et al.*, 1992), and sorghum (Casa *et al.*, 1993; Hagio *et al.*, 1991); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Buising and Benbow, 1994), soybean (U.S. Patent No. 5,322,783, specifically incorporated

herein by reference in its entirety), sunflower (Knittel *et al.* 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.* 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

5 (iii) *Agrobacterium-mediated Transformation*

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley *et al.*, (1985), Rogers *et al.*, (1987) and U.S. Patent
10 No. 5,563,055, specifically incorporated herein by reference in its entirety.

Agrobacterium-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with
15 dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.*, 1997; Zhang *et al.*, 1997; U.S. Patent No. 5,591,616, specifically incorporated herein by
20 reference in its entirety), wheat (McCormac *et al.*, 1998), barley (Tingay *et al.*, 1997; McCormac *et al.*, 1998), and maize (Ishidia *et al.*, 1996), U.S. Patent No. 5,591,616; European Patent Application EP 604 662.

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985).
25 Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable
30 for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes

can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

(iv) *Other Transformation Methods*

5 Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, e.g., Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

10 Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts have been described (Fujimara *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 1993 and U.S. Patent No. 5,508,184; each specifically incorporated herein by reference in its entirety). Examples of the use of direct uptake transformation of cereal protoplasts include transformation of rice (Ghosh-
15 Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also,
20 silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppeler, 1990; Kaeppeler *et al.*, 1992; U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cell are punctured. This technique has been used successfully with, for example, the monocot
25 cereals maize (PCT Application WO 95/06128, specifically incorporated herein by reference in its entirety; Thompson, 1995) and rice (Nagatani, 1997).

VI. Optimization of Microprojectile Bombardment

For microprojectile bombardment transformation in accordance with the current invention, both physical and biological parameters may be optimized. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, such as the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, the orientation of an immature embryo or other target tissue relative to the particle trajectory, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as DNA concentration, gap distance, flight distance, tissue distance, and helium pressure. It is further contemplated that the grade of helium may effect transformation efficiency. For example, differences in transformation efficiencies may be witnessed between bombardments using industrial grade (99.99% pure) or ultra pure helium (99.999% pure), although it is not currently clear which is more advantageous for use in bombardment. One also may optimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation.

(i) *Physical Parameters*

1. Gap Distance

The variable nest (macro holder) can be adjusted to vary the distance between the rupture disk and the macroprojectile, *i.e.*, the gap distance. This distance can be varied from 0 to 2 cm.

The predicted effects of a shorter gap are an increase of velocity of both the macro- and microprojectiles, an increased shock wave (which leads to tissue splattering and increased tissue trauma), and deeper penetration of microprojectiles. Longer gap distances would have the

opposite effects but may increase viability and therefore the total number of recovered stable transformants.

2. Flight Distance

5 The fixed nest (contained within the variable nest) can be varied between 0.5 and 2.25 cm in predetermined 0.5 cm increments by the placement of spacer rings to adjust the flight path traversed by the macroprojectile. Short flight paths allow for greater stability of the macroprojectile in flight but reduce the overall velocity of the microprojectiles. Increased stability in flight increases, for example, the number of centered GUS foci. Greater flight
10 distances (up to some point) increase velocity but also increase instability in flight. Based on observations, it is recommended that bombardments typically be done with a flight path length of about 1.0 cm to 1.5 cm.

3. Tissue Distance

15 Placement of tissue within the gun chamber can have significant effects on microprojectile penetration. Increasing the flight path of the microprojectiles will decrease velocity and trauma associated with the shock wave. A decrease in velocity also will result in shallower penetration of the microprojectiles.

4. Helium Pressure

20 By manipulation of the type and number of rupture disks, pressure can be varied between 400 and 2000 psi within the gas acceleration tube. Optimum pressure for stable transformation has been determined to be between 1000 and 1200 psi.

5. Coating of Microprojectiles.

25 For microprojectile bombardment, one will attach (*i.e.* "coat") DNA to the microprojectiles such that it is delivered to recipient cells in a form suitable for transformation thereof. In this respect, at least some of the transforming DNA must be available to the target cell for transformation to occur, while at the same time during delivery the DNA must be
30 attached to the microprojectile. Therefore, availability of the transforming DNA from the microprojectile may comprise the physical reversal of bonds between transforming DNA and the microprojectile following delivery of the microprojectile to the target cell. This need not be the

case, however, as availability to a target cell may occur as a result of breakage of unbound segments of DNA or of other molecules which comprise the physical attachment to the microprojectile. Availability may further occur as a result of breakage of bonds between the transforming DNA and other molecules, which are either directly or indirectly attached to the microprojectile. It further is contemplated that transformation of a target cell may occur by way of direct recombination between the transforming DNA and the genomic DNA of the recipient cell. Therefore, as used herein, a "coated" microprojectile will be one which is capable of being used to transform a target cell, in that the transforming DNA will be delivered to the target cell, yet will be accessible to the target cell such that transformation may occur.

Any technique for coating microprojectiles which allows for delivery of transforming DNA to the target cells may be used. Methods for coating microprojectiles which have been demonstrated to work well with the current invention have been specifically disclosed herein. DNA may be bound to microprojectile particles using alternative techniques, however. For example, particles may be coated with streptavidin and DNA end labeled with long chain thiol cleavable biotinylated nucleotide chains. The DNA adheres to the particles due to the streptavidin-biotin interaction, but is released in the cell by reduction of the thiol linkage through reducing agents present in the cell.

Alternatively, particles may be prepared by functionalizing the surface of a gold oxide particle, providing free amine groups. DNA, having a strong negative charge, binds to the functionalized particles. Furthermore, charged particles may be deposited in controlled arrays on the surface of mylar flyer disks used in the PDS-1000 Biolistics device, thereby facilitating controlled distribution of particles delivered to target tissue.

As disclosed above, it further is proposed, that the concentration of DNA used to coat microprojectiles may influence the recovery of transformants containing a single copy of the transgene. For example, a lower concentration of DNA may not necessarily change the efficiency of the transformation, but may instead increase the proportion of single copy insertion events. In this regard, approximately 1 ng to 2000 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles. In other embodiments of the invention, approximately 2.5 ng to 1000

ng, 2.5 ng to 750 ng, 2.5 ng to 500 ng, 2.5 ng to 250 ng, 2.5 ng to 100 ng, or 2.5 ng to 50 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles.

Various other methods also may be used to increase transformation efficiency and/or
5 increase the relative proportion of low-copy transformation events. For example, the inventors contemplate end-modifying transforming DNA with alkaline phosphatase or an agent which will blunt DNA ends prior to transformation. Still further, an inert carrier DNA may be included with the transforming DNA, thereby lowering the effective transforming DNA concentration without lowering the overall amount of DNA used. These techniques are further described in U.S. Patent
10 Application Serial No. 08/995,451, filed December 22, 1997, the disclosure of which is specifically incorporated herein by reference in its entirety.

(ii) Biological Parameters

Culturing conditions and other factors can influence the physiological state of the target
15 cells and may have profound effects on transformation and integration efficiencies. First, the act of bombardment could stimulate the production of ethylene which could lead to senescence of the tissue. The addition of antiethylene compounds could increase transformation efficiencies. Second, it is proposed that certain points in the cell cycle may be more appropriate for integration of introduced DNA. Hence synchronization of cell cultures may enhance the frequency of
20 production of transformants. For example, synchronization may be achieved using cold treatment, amino acid starvation, or other cell cycle-arresting agents. Third, the degree of tissue hydration also may contribute to the amount of trauma associated with bombardment as well as the ability of the microprojectiles to penetrate cell walls.

25 The position and orientation of an embryo or other target tissue relative to the particle trajectory also may be important. For example, the PDS-1000 biolistics device does not produce a uniform spread of particles over the surface of a target petri dish. The velocity of particles in the center of the plate is higher than the particle velocity at further distances from the center of the petri dish. Therefore, it is advantageous to situate target tissue on the petri dish such as to
30 avoid the center of the dish, referred to by some as the "zone of death." Furthermore, orientation of the target tissue with regard to the trajectory of targets also can be important. It is contemplated that it is desirable to orient the tissue most likely to regenerate a plant toward the

particle stream. For example, the scutellum of an immature embryo comprises the cells of greatest embryogenic potential and therefore should be oriented toward the particle stream.

It also has been reported that slightly plasmolyzed yeast cells allow increased transformation efficiencies (Armaleo *et al.*, 1990). It was hypothesized that the altered osmotic state of the cells helped to reduce trauma associated with the penetration of the microprojectile. Additionally, the growth and cell cycle stage may be important with respect to transformation.

1. Osmotic Adjustment

It has been suggested that osmotic pre-treatment could potentially reduce bombardment associated injury as a result of the decreased turgor pressure of the plasmolyzed cell. In a previous study, the number of cells transiently expressing GUS increased following subculture into both fresh medium and osmotically adjusted medium (PCT Application WO 95/06128, specifically incorporated herein by reference in its entirety). Pretreatment times of 90 minutes showed higher numbers of GUS expressing foci than shorter times. Cells incubated in 500 mOSM/kg medium for 90 minutes showed an approximately 3.5 fold increase in transient GUS foci than the control. Preferably, immature embryos are precultured for 4-5 hours prior to bombardment on culture medium containing 12% sucrose. A second culture on 12% sucrose is performed for 16-24 hours following bombardment. Alternatively, type II cells are pretreated on 0.2M mannitol for 3-4 hours prior to bombardment. It is contemplated that pretreatment of cells with other osmotically active solutes for a period of 1-6 hours also may be desirable.

2. Plasmid Configuration

In some instances, it will be desirable to deliver DNA to maize cells that does not contain DNA sequences necessary for maintenance of the plasmid vector in the bacterial host, *e.g.*, *E. coli*, such as antibiotic resistance genes, including but not limited to ampicillin, kanamycin, and tetracycline resistance, and prokaryotic origins of DNA replication. In such case, a DNA fragment containing the transforming DNA may be purified prior to transformation. An exemplary method of purification is gel electrophoresis on a 1.2% low melting temperature agarose gel, followed by recovery from the agarose gel by melting gel slices in a 6-10 fold excess of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 70°C-72°C); frozen and thawed (37°C); and the agarose pelleted by centrifugation. A Qiagen Q-100 column then may be used

for purification of DNA. For efficient recovery of DNA, the flow rate of the column may be adjusted to 40 ml/hr.

Isolated DNA fragments can be recovered from agarose gels using a variety of electroelution techniques, enzyme digestion of the agarose, or binding of DNA to glass beads (e.g., Gene Clean). In addition, HPLC and/or use of magnetic particles may be used to isolate DNA fragments. As an alternative to isolation of DNA fragments, a plasmid vector can be digested with a restriction enzyme and this DNA delivered to maize cells without prior purification of the expression cassette fragment.

VII. Recipient Cells for Transformation

Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium usually is a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, plant cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures typically requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH, but also by whether media is solid or liquid. Table 7 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

Recipient cell targets include, but are not limited to, meristem cells, Type I, Type II, and Type III callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. It is contemplated that any cell from which a fertile plant may be regenerated is useful as a recipient cell. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Those cells which are capable of proliferating as callus also are recipient cells for genetic transformation. The present invention provides techniques for transforming immature embryos and subsequent regeneration of fertile transgenic plants. Transformation of immature embryos obviates the need for long term development of recipient cell cultures. Pollen, as well as its precursor cells, microspores, may be capable of functioning as recipient cells for genetic transformation, or as vectors to carry foreign DNA for incorporation during fertilization. Direct pollen transformation would obviate the need for cell culture. Meristematic cells (*i.e.*, plant cells capable of continual cell division and characterized by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, *etc.*) may represent another type of recipient plant cell. Because of their undifferentiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an *in vitro* meristematic cell system, retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

Cultured plant cells that can serve as recipient cells for transforming with desired DNA segments may be any plant cells including corn cells, and more specifically, cells from *Zea mays* L. Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. An example of non-embryogenic cells are certain Black Mexican Sweet (BMS) corn cells.

The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, *e.g.*, as recipient cells for transformation, has been described in U.S. Pat. No. 5,134,074; U.S. Patent No. 5,538,877 and U.S. Pat. No. 5,489,520; each of which is incorporated herein by reference in its entirety.

Certain techniques may be used that enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of recipient cells for use in, microprojectile transformation. Suspension culturing, particularly using the media disclosed herein, may improve the ratio of recipient to non-recipient cells in any given population. Manual selection techniques which can be employed to select recipient cells may include, *e.g.*, assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation also is a possible method of selecting for recipient cells.

Manual selection of recipient cells, *e.g.*, by selecting embryogenic cells from the surface of a Type II callus, is one means that may be used in an attempt to enrich for recipient cells prior to culturing (whether cultured on solid media or in suspension). The preferred cells may be those located at the surface of a cell cluster, and may further be identifiable by their lack of differentiation, their size and dense cytoplasm. The preferred cells will generally be those cells which are less differentiated, or not yet committed to differentiation. Thus, one may wish to identify and select those cells which are cytoplasmically dense, relatively unvacuolated with a high nucleus to cytoplasm ratio (*e.g.*, determined by cytological observations), small in size (*e.g.*, 10-20 μm), and capable of sustained divisions and somatic proembryo formation.

It is proposed that other means for identifying such cells also may be employed. For example, through the use of dyes, such as Evan's blue, which are excluded by cells with relatively non-permeable membranes, such as embryogenic cells, and taken up by relatively differentiated cells such as root-like cells and snake cells (so-called due to their snake-like appearance).

Other possible means of identifying recipient cells include the use of isozyme markers of embryogenic cells, such as glutamate dehydrogenase, which can be detected by cytochemical stains (Fransz *et al.*, 1989). However, it is cautioned that the use of isozyme markers including glutamate dehydrogenase may lead to some degree of false positives from non-embryogenic cells such as rooty cells which nonetheless have a relatively high metabolic activity.

It is anticipated that the A3 promoter and/or actin 2 intron enhancer may be useful for regulating gene expression in dicot plants. Identification of recipient cells and transformation of

many dicot species have been reported. For example, leaf segments of *Nicotiana tabacum* or *Lycopersicon esculentum* may be transformed by microprojectile bombardment using *Agrobacterium tumefaciens*. In some dicot species, recipient cells are found in germinating seedlings. For example, hypocotyls and cotyledons of cotton and soybean seedlings may be used
5 to produce transformants using either *Agrobacterium tumefaciens* or microprojectile bombardment. It is contemplated that, in both monocot and dicot plant species, transformation may be achieved utilizing any target cell population from which a fertile plant may be regenerated. It further is anticipated that transformation of some plant species may be achieved without tissue culture. For example, *Arabidopsis thaliana* may be transformed by dipping the
10 inflorescence in a culture of *Agrobacterium tumefaciens* under conditions of reduced atmospheric pressure (Chang *et al.*, 1994).

(i) *Culturing Cells to be Recipients for Transformation*

The ability to prepare and cryopreserve cultures of plant cells is important to certain
15 aspects of the present invention, in that it provides a means for reproducibly and successfully preparing cells for transformation. A variety of different types of media have been previously developed and may be employed in carrying out various aspects of the invention. The following table, Table 7, sets forth the composition of the media preferred by the inventor for carrying out these aspects of the invention.

Table 7

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
7	MS*	2%	6.0	.25 mg thiamine .5 mg BAP .5 mg NAA Bactoagar
10	MS	2%	6.0	.25 mg thiamine 1 mg BAP 1 mg 2,4-D 400 mg L-proline Bactoagar
19	MS	2%	6.0	.25 mg thiamine .25 mg BAP .25 mg NAA Bactoagar
20	MS	3%	6.0	.25 mg thiamine 1 mg BAP 1 mg NAA Bactoagar
52	MS	2%	6.0	.25 mg thiamine 1 mg 2,4-D 10 ⁻⁷ M ABA BACTOAGAR
101	MS	3%	6.0	MS vitamins 100 mg myo-inositol Bactoagar

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
105	MS	--	3.5	0.04 mg NAA 3mg BAP 1mg thiamine.HCl 0.5 mg niacin 0.91 mg L-asparagine monohydrate 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline 20 g sorbitol 2.0 g Gelgro
110	MS	6%	5.8	1 mg thiamine.HCl 1 mg niacin 3.6 g Gelgro
163	MS	3%	6.0	MS vitamins 3.3 mg dicamba 100 mg myo-inositol Bactoagar
171	MS	3%	6.0	MS vitamins .25 mg 2,4-D 10 mg BAP 100 mg myo-inositol Bactoagar

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
173	MS	6%	6.0	MS vitamins 5 mg BAP .186 mg NAA .175 mg IAA .403 mg 2IP 10^{-7} M ABA 200 mg myo-inositol Bactoagar
177	MS	3%	6.0	MS vitamins .25 mg 2,4-D 10 mg BAP 10^{-7} M ABA 100 mg myo-inositol Bactoagar
185	MS	-	5.8	3 mg BAP .04 mg NAA RT vitamins 1.65 mg thiamine 1.38 g L-proline 20 g sorbitol Bactoagar
189	MS	-	5.8	3 mg BAP .04 mg NAA .5 mg niacin 800 mg L-asparagine 100 mg casamino acids 20 g sorbitol 1.4 g L-proline 100 mg myo-inositol Gelgro

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
201	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1 mg 2,4-D 100 mg casein hydrolysate 2.9 g L-proline Gelgro
205	N6	2%	5.8	N6 vitamins 2 mg L-glycine .5 mg 2,4-D 100 mg casein hydrolysate 2.9 g L-proline Gelgro
209	N6	6%	5.8	N6 vitamins 2 mg L-glycine 100 mg casein hydrolysate 0.69 g L-proline Bactoagar

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
210	N6	3%	5.5	N6 vitamins 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo-inositol 790 mg L-asparagine 100 mg casein hydrolysate 1.4 g L-proline Hazelton agar**** 2 mg L-glycine
212	N6	3%	5.5	N6 vitamins 2 mg L-glycine 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline Hazelton agar****
227	N6	2%	5.8	N6 vitamins 2 mg L-glycine 13.2 mg dicamba 100 mg casein hydrolysate 2.9 g L-proline Gelgro

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
273 (also, 201V, 236S, 201D, 2071, 2366, 201SV, 2377, and 201BV)	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1 mg 2,4-D 16.9 mg AgNO ₃ 100 mg casein hydrolysate 2.9 g L-proline
279	N6	2%	5.8	3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L-asparagine 100 mg casein hydrolysate 100 mg myoinositol 1.4 g L-proline Gelgro****
288	N6	3%		3.3 mg dicamba 1 mg thiamine .5 mg niacin .8 g L-asparagine 100 mg myo-inositol 1.4 g L-proline 100 mg casein hydrolysate 16.9 mg AgNO ₃ Gelgro

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
401	MS	3%	6.0	3.73 mg Na ₂ EDTA .25 mg thiamine 1 mg 2,4-D 2 mg NAA 200 mg casein hydrolysate 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol
402	MS	3%	6.0	3.73 mg Na ₂ EDTA .25 mg thiamine 1 mg 2,4-D 200 mg casein hydrolysate 2.9 g L-proline 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol
409	MS	3%	6.0	3.73 mg Na ₂ EDTA .25 mg thiamine 9.9 mg dicamba 200 mg casein hydrolysate 2.9 g L-proline 500 mg K ₂ SO ₄ 400 mg KH ₂ PO ₄ 100 mg myo-inositol
501	Clark's Medium***	2%	5.7	
607	1/2 x MS	3%	5.8	1 mg thiamine 1 mg niacin Gelrite

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
615	MS	3%	6.0	MS vitamins 6 mg BAP 100 mg myo-inositol Bactoagar
617	1/2 x MS	1.5%	6.0	MS vitamins 50 mg myo-inositol Bactoagar
708	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 200 mg casein hydrolysate 0.69 g L-proline Gelrite
721	N6	2%	5.8	3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L-asparagine 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline 54.65 g mannitol Gelgro

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
726	N6	3%	5.8	3.3 mg dicamba .5 mg niacin 1 mg thiamine 800 mg L-asparagine 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline
727	N6	3%	5.8	N6 vitamins 2 mg L-glycine 9.9 mg dicamba 100 mg casein hydrolysate 2.9 g L-proline Gelgro
728	N6	3%	5.8	N6 vitamins 2 mg L-glycine 9.9 mg dicamba 16.9 mg AgNO ₃ 100 mg casein hydrolysate 2.9 g L-proline Gelgro

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
734	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 14 g Fe sequestrene (replaces Fe-EDTA) 200 mg casein hydrolyste 0.69 g L-proline Gelrite
735	N6	2%	5.8	1 mg 2,4-D .5 mg niacin .91 g L-asparagine 100 mg myo-inositol 1 mg thiamine .5 g MES .75 g MgCl ₂ 100 mg casein hydrolysate 0.69 g L-proline Gelgro
2004	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 17 mg AgNO ₃ 1.4 g L-proline 0.8 g L-asparagine 100 mg casein hydrolysate 100 mg myo-inositol Gelrite

Table 7 - Continued

Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
2008	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 1.4 g L-proline 0.8 g L-asparagine Gelrite

- 5 *Basic MS medium described in Murashige and Skoog (1962). This medium is typically modified by decreasing the NH_4NO_3 from 1.64 g/l to 1.55 g/l, and omitting the pyridoxine HCl, nicotinic acid, myo-inositol and glycine.

** NAA = Naphthol Acetic Acid

10 IAA = Indole Acetic Acid

2-IP = 2, isopentyl adenine

2,4-D = 2, 4-Dichlorophenoxyacetic Acid

BAP = 6-Benzyl aminopurine

ABA = abscisic acid

15 *** Basic medium described in Clark (1982)

**** These media may be made with or without solidifying agent.

A number of exemplary maize cultures which may be used for transformation have been developed and are disclosed in U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993, which is
20 specifically incorporated herein by reference.

(ii) *Media*

In certain embodiments of the current invention, recipient cells may be selected following growth in culture. Where employed, cultured cells may be grown either on solid supports or in

the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components (see Table 7), but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.* (1975) and MS media (Murashige and Skoog, 1962). It has been discovered that media such as MS which have a high ammonia/nitrate ratio are counterproductive to the generation of recipient cells in that they promote loss of morphogenic capacity. N6 media, on the other hand, has a somewhat lower ammonia/nitrate ratio, and is contemplated to promote the generation of recipient cells by maintaining cells in a proembryonic state capable of sustained divisions.

(iii) Maintenance

The method of maintenance of cell cultures may contribute to their utility as sources of recipient cells for transformation. Manual selection of cells for transfer to fresh culture medium, frequency of transfer to fresh culture medium, composition of culture medium, and environmental factors including, but not limited to, light quality and quantity and temperature are all important factors in maintaining callus and/or suspension cultures that are useful as sources of recipient cells. It is contemplated that alternating callus between different culture conditions may be beneficial in enriching for recipient cells within a culture. For example, it is proposed that cells may be cultured in suspension culture, but transferred to solid medium at regular intervals. After a period of growth on solid medium cells can be manually selected for return to liquid culture medium. It is proposed that by repeating this sequence of transfers to fresh culture medium it is possible to enrich for recipient cells. It also is contemplated that passing cell cultures through a 1.9 mm sieve is useful in maintaining the friability of a callus or suspension culture and may be beneficial in enriching for transformable cells.

(iv) *Cryopreservation Methods*

Cryopreservation is important because it allows one to maintain and preserve a known transformable cell culture for future use, while eliminating the cumulative detrimental effects associated with extended culture periods.

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Cell suspensions and callus were cryopreserved using modifications of methods previously reported (Finkle, 1985; Withers & King, 1979). The cryopreservation protocol comprised adding a pre-cooled (0°C) concentrated cryoprotectant mixture stepwise over a period of one to two hours to pre-cooled (0°C) cells. The mixture was maintained at 0°C throughout this period. The volume of added cryoprotectant was equal to the initial volume of the cell suspension (1:1 addition), and the final concentration of cryoprotectant additives was 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 MW), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at 0°C for 30 minutes, during which time the cell suspension/cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.5°C/minute to -8°C and held at this temperature for ice nucleation.

Once extracellular ice formation had been visually confirmed, the tubes were cooled at 0.5°C/minute from -8°C to -35°C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout the cell clusters). At this point, the cells had lost the majority of their osmotic volume (*i.e.*, there is little free water left in the cells), and they could be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in the cells in conjunction with the rapid cooling rates from -35°C to -196°C prevented large organized ice crystals from forming in the cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to the point where long-term storage should not be detrimental.

Thawing of the extracellular solution was accomplished by removing the cryo-tube from liquid nitrogen and swirling it in sterile 42°C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter, resting on a layer of BMS cells (the feeder layer which provided a nurse effect during recovery). The

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cryoprotectant solution is removed by pipetting. Culture medium comprised a callus proliferation medium with increased osmotic strength. Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to the recovering cells. Once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. If initiation of a suspension culture was desired, the cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks). Alternatively, cells were cultured on solid callus proliferation medium. After the culture was reestablished in liquid (within 1 to 2 additional weeks), it was used for transformation experiments. When desired, previously cryopreserved cultures may be frozen again for storage.

VIII. Production and Characterization of Stably Transformed Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. As mentioned herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

(i) *Selection*

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphotransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Using the techniques disclosed herein, greater than 40% of bombarded embryos may yield transformants.

One herbicide which constitutes a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block, 1987) *Brassica* (De Block, 1989) and maize (U.S. Pat. No. 5,550,318). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Pat. No. 4,535,060 describes the

isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced *in vitro*. Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other EPSPS genes will also be useful (PCT/WO97/4103).

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, bombarded tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility in the practice of the invention. Tissue can be placed on any porous, inert, solid or semi-solid support for bombardment, including but not limited to filters and solid culture medium. Bialaphos and glyphosate are provided as examples of agents suitable for selection of transformants, but the technique of this invention is not limited to them.

It is further contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993; U.S. Pat. No. 5,508,468; U.S. Patent No. 5,780,708; and U.S. Pat. No. 5,508,468; each of the disclosures of which is specifically incorporated herein by reference in its entirety).

Alternatively, a gene encoding anthranilate synthase, which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Pat. No. 5,508,468; and PCT Publication WO 97/26366.

It is further contemplated that genes conferring resistance to antibiotics may be useful as selectable marker genes. For example, expression of the amino glycoside phosphotransferase II

(aptII; also known as neomycin phosphotransferase II, nptII) gene confers resistance to aminoglycoside antibiotics including kanamycin, neomycin, G418 and paramomycin. Expression of a gene encoding hygromycin phosphotransferase confers resistance to hygromycin. It is anticipated that genes conferring resistance to antibiotics other than those listed herein are known in the art and may be useful as selectable marker genes.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media. The R-locus is useful for selection of transformants from bombarded immature embryos. In a similar fashion, the introduction of the C1 and B genes will result in pigmented cells and/or tissues.

The enzyme luciferase may be used as a screenable marker in the context of the present invention. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another screenable marker which may be used in a similar fashion is the gene coding for green fluorescent protein.

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover

transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types.

5 (ii) *Regeneration and Seed Production*

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified (see Table 7) by including further substances such as growth regulators. A preferred growth regulator for such purposes is
10 dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and like ways has been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is
15 suitable for regeneration, at least 2 wk, then transferred to media conducive to maturation of embryoids. Cultures are transferred every 2 wk on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate
20 medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soilless plant growth mix, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO₂, and 25-250 microeinsteins m⁻² s⁻¹ of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants are regenerated from about 6 wk to 10 months after a transformant is identified, depending on the
25 initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes and Plant Cons. Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

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Note, however, that seeds on transformed plants may occasionally require embryo rescue due to cessation of seed development and premature senescence of plants. To rescue developing

embryos, they are excised from surface-disinfected seeds 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that may be
5 cultured for 1 wk on media containing the above ingredients along with 10^{-5} M abscisic acid and then transferred to growth regulator-free medium for germination.

Progeny may be recovered from transformed plants and tested for expression of the exogenous expressible gene by localized application of an appropriate substrate to plant parts
10 such as leaves. In the case of *bar* transformed plants, it was found that transformed parental plants (R_0) and their progeny of any generation tested exhibited no bialaphos-related necrosis after localized application of the herbicide Basta to leaves, if there was functional PAT activity in the plants as assessed by an *in vitro* enzymatic assay. All PAT positive progeny tested contained *bar*, confirming that the presence of the enzyme and the resistance to bialaphos were associated
15 with the transmission through the germline of the marker gene.

(iii) Characterization

To confirm the presence of the exogenous DNA or "transgene(s)" in the regenerating plants, a variety of assays may be performed. Such assays include, for example, "molecular
20 biological" assays, such as Southern and Northern blotting and PCRTM; "biochemical" assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

1. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from callus cell lines or any plant parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.
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The presence of DNA elements introduced through the methods of this invention may be determined by polymerase chain reaction (PCRTM). Using this technique discreet fragments of

DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. It is the experience of the inventor, however, that DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR™ analysis. In addition, it is not possible using PCR™ techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR™ techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR™, *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR™, *e.g.*, the presence of a gene.

Both PCR™ and Southern hybridization techniques can be used to demonstrate transmission of a transgene to progeny. In most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer *et al.*, 1992) indicating stable inheritance of the transgene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be

necessary to prepare RNA for analysis from these tissues. PCR™ techniques also may be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR™ it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR™ techniques amplify the DNA. In most instances PCR™ techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species also can be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

2. Gene Expression

While Southern blotting and PCR™ may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying

the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and ^{14}C -acetyl CoA or for anthranilate synthase activity by following loss of
5 fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological
10 properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are
15 evaluated under carefully controlled conditions termed bioassays.

IX. Site Specific Integration or Excision of Transgenes

It is specifically contemplated by the inventors that one could employ techniques for the site-specific integration or excision of transformation constructs prepared in accordance with the
20 instant invention. An advantage of site-specific integration or excision is that it can be used to overcome problems associated with conventional transformation techniques, in which transformation constructs typically randomly integrate into a host genome in multiple copies. This random insertion of introduced DNA into the genome of host cells can be lethal if the foreign DNA inserts into an essential gene. In addition, the expression of a transgene may be
25 influenced by "position effects" caused by the surrounding genomic DNA. Further, because of difficulties associated with plants possessing multiple transgene copies, including gene silencing, recombination and unpredictable inheritance, it is typically desirable to control the copy number of the inserted DNA, often only desiring the insertion of a single copy of the DNA sequence.

Site-specific integration or excision of transgenes or parts of transgenes can be achieved
30 in plants by means of homologous recombination (see, for example, U.S. Patent No. 5,527,695, specifically incorporated herein by reference in its entirety). Homologous recombination is a

reaction between any pair of DNA sequences having a similar sequence of nucleotides, where the two sequences interact (recombine) to form a new recombinant DNA species. The frequency of homologous recombination increases as the length of the shared nucleotide DNA sequences increases, and is higher with linearized plasmid molecules than with circularized plasmid molecules. Homologous recombination can occur between two DNA sequences that are less than identical, but the recombination frequency declines as the divergence between the two sequences increases.

Introduced DNA sequences can be targeted via homologous recombination by linking a DNA molecule of interest to sequences sharing homology with endogenous sequences of the host cell. Once the DNA enters the cell, the two homologous sequences can interact to insert the introduced DNA at the site where the homologous genomic DNA sequences were located. Therefore, the choice of homologous sequences contained on the introduced DNA will determine the site where the introduced DNA is integrated via homologous recombination. For example, if the DNA sequence of interest is linked to DNA sequences sharing homology to a single copy gene of a host plant cell, the DNA sequence of interest will be inserted via homologous recombination at only that single specific site. However, if the DNA sequence of interest is linked to DNA sequences sharing homology to a multicopy gene of the host eukaryotic cell, then the DNA sequence of interest can be inserted via homologous recombination at each of the specific sites where a copy of the gene is located.

DNA can be inserted into the host genome by a homologous recombination reaction involving either a single reciprocal recombination (resulting in the insertion of the entire length of the introduced DNA) or through a double reciprocal recombination (resulting in the insertion of only the DNA located between the two recombination events). For example, if one wishes to insert a foreign gene into the genomic site where a selected gene is located, the introduced DNA should contain sequences homologous to the selected gene. A single homologous recombination event would then result in the entire introduced DNA sequence being inserted into the selected gene. Alternatively, a double recombination event can be achieved by flanking each end of the DNA sequence of interest (the sequence intended to be inserted into the genome) with DNA sequences homologous to the selected gene. A homologous recombination event involving each of the homologous flanking regions will result in the insertion of the foreign DNA. Thus only

those DNA sequences located between the two regions sharing genomic homology become integrated into the genome.

Although introduced sequences can be targeted for insertion into a specific genomic site via homologous recombination, in higher eukaryotes homologous recombination is a relatively rare event compared to random insertion events. In plant cells, foreign DNA molecules find homologous sequences in the cell's genome and recombine at a frequency of approximately $0.5\text{--}4.2 \times 10^{-4}$. Thus any transformed cell that contains an introduced DNA sequence integrated via homologous recombination will also likely contain numerous copies of randomly integrated introduced DNA sequences. Therefore, to maintain control over the copy number and the location of the inserted DNA, these randomly inserted DNA sequences can be removed. One manner of removing these random insertions is to utilize a site-specific recombinase system. In general, a site specific recombinase system consists of three elements: two pairs of DNA sequence (the site - specific recombination sequences) and a specific enzyme (the site-specific recombinase). The site-specific recombinase will catalyze a recombination reaction only between two site -specific recombination sequences.

A number of different site specific recombinase systems could be employed in accordance with the instant invention, including, but not limited to, the Cre/lox system of bacteriophage P1 (U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety), the FLP/FRT system of yeast (Golic and Lindquist, 1989; U.S. Patent No. 5,744,336), the Gin recombinase of phage Mu (Maeser *et al.*, 1991), the Pin recombinase of *E. coli* (Enomoto *et al.*, 1983), and the R/RS system of the pSR1 plasmid (Araki *et al.*, 1992). The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems constitute two particularly useful systems for site specific integration or excision of transgenes. In these systems a recombinase (Cre or FLP) will interact specifically with its respective site -specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT) and therefore, convenient for use with transformation vectors.

The FLP/FRT recombinase system has been demonstrated to function efficiently in plant cells (U.S. Patent No. 5,744,386. Experiments on the performance of the FLP/FRT system in both maize and rice protoplasts indicate that FRT site structure, and amount of the FLP protein

present, affects excision activity. In general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The systems can catalyze both intra- and intermolecular reactions in maize protoplasts, indicating its utility for DNA excision as well as integration reactions. The recombination reaction is reversible and this
5 reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site - specific recombination sequences is one approach to remedying this situation. The site -specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

10 In the Cre-lox system, discovered in bacteriophage P1, recombination between loxP sites occurs in the presence of the Cre recombinase (see, *e.g.*, U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety). This system has been utilized to excise a gene located between two lox sites which had been introduced into a yeast genome (Sauer, 1987). Cre was expressed from an inducible yeast GAL1 promoter and this Cre gene was located on an
15 autonomously replicating yeast vector.

Since the lox site is an asymmetrical nucleotide sequence, lox sites on the same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA
20 molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA
25 molecules can occur. All of these recombination events are catalyzed by the product of the Cre coding region.

X. Breeding Plants of the Invention

In addition to direct transformation of a particular genotype with a construct prepared
30 according to the current invention, plants of the invention may be made by crossing a plant

having a construct of the invention to a second, plant lacking the construct. For example, a transgene comprising an actin 2 intron and/or an actin 2 promoter can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the current invention not only encompasses a plant directly regenerated
5 from cells which have been transformed in accordance with the current invention, but also the progeny of such plants. As used herein the term "progeny" denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a construct prepared in accordance with the invention. "Crossing" a plant to provide a plant line having one or more added transgenes relative to a starting plant line, as
10 disclosed herein, is defined as the techniques that result in a transgene of the invention being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene of the invention. To achieve this one would, generally, perform the following steps:

(a) plant seeds of the first (starting line) and second (donor plant line that comprises a transgene of the invention) parent plants;

15 (b) grow the seeds of the first and second parent plants into plants that bear flowers;

(c) pollinate the female flower of the first parent plant with the pollen of the second parent plant; and

(d) harvest seeds produced on the parent plant bearing the female flower.

Backcrossing is herein defined as the process including the steps of:

20 (a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking said desired gene, DNA sequence or element;

(b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;

(c) crossing the progeny plant to a plant of the second genotype; and

25 (d) repeating steps (b) and (c) for the purpose of transferring said desired gene, DNA sequence or element from a plant of a first genotype to a plant of a second genotype.

Introgression of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been
30 introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking said desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

XI. Definitions

Genetic Transformation: A process of introducing a DNA sequence or construct (*e.g.*, a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

Exogenous gene: A gene which is not normally present in a given host genome in the exogenous gene's present form. In this respect, the gene itself may be native to the host genome, however, the exogenous gene will comprise the native gene altered by the addition or deletion of one or more different regulatory elements.

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

Expression cassette: A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred expression cassettes will comprise all of the genetic elements necessary to direct the expression of a selected gene.

Expression vector: A vector comprising an expression cassette.

Progeny: Any subsequent generation, including the seeds and plants therefrom, which is derived from a particular parental plant or set of parental plants.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

R₀ Transgenic Plant: A plant which has been directly transformed with a selected DNA or has been regenerated from a cell or cell cluster which has been transformed with a selected DNA.

Regeneration: The process of growing a plant from a plant cell (*e.g.*, plant protoplast, callus or explant).

Selected DNA: A DNA which one desires to have expressed in a transgenic plant, plant cell or plant part. A selected DNA may be native or foreign to a host genome, but where the selected DNA is present in the host genome, may include one or more regulatory or functional elements which alter the expression profile of the selected gene relative to native copies of the gene.

Selected Gene: A gene which one desires to have expressed in a transgenic plant, plant cell or plant part. A selected gene may be native or foreign to a host genome, but where the selected gene is present in the host genome, will include one or more regulatory or functional elements which differ from native copies of the gene.

5 **Transformation construct:** A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred transformation constructs will comprise all of the genetic elements necessary to direct the expression of one or more exogenous genes. Transformation constructs prepared in accordance with the instant invention will include an actin 2 intron and/or an actin 2 promoter. In particular embodiments of the instant
10 invention, it may be desirable to introduce a transformation construct into a host cell in the form of an expression cassette.

Transformed cell: A cell the DNA complement of which has been altered by the introduction of an exogenous DNA molecule into that cell.

Transgene: A segment of DNA which has been incorporated into a host genome or is
15 capable of autonomous replication in a host cell and is capable of causing the expression of one or more cellular products. Exemplary transgenes will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant. Transgenes may be directly introduced into a plant by genetic transformation, or may be inherited from a plant of any previous generation which was transformed with the DNA segment.

20 **Transgenic plant:** A plant or progeny plant of any subsequent generation derived therefrom, wherein the DNA of the plant or progeny thereof contains an introduced exogenous DNA segment not originally present in a non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the "exogenous" gene has been altered in order to alter the level or pattern of expression
25 of the gene.

Transit peptide: A polypeptide sequence which is capable of directing a polypeptide to a particular organelle or other location within a cell.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment.

30 A plasmid is an exemplary vector.

XII. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1

Cloning of the Rice Actin 2 Promoter and Intron Sequences

Two plasmid clones (pUC-*Rac2* and pUC-*Rac4*) containing genomic DNA sequences of the rice actin 2 gene (*Act2*) were isolated as described in Reece *et al.*, 1990. Restriction maps were generated for each clone, which consisted of an *EcoRI* restriction fragment from rice genomic DNA cloned in pUC13. The location of the region 5' of the translation start codon for in each of these clones was determined by comparing their restriction maps with those determined by sequence analysis of the pUC-*Rac2* coding region (Reece *et al.*, 1990). Restriction mapping and preliminary sequence analysis indicated that the pUC-*Rac4* genomic clone was identical to, but 1.2 kb longer than, that of the pUC-*Rac2* clone.

Sequence characterization was carried out to determine the length and structure of the 5' region in pUC-*Rac2* and pUC-*Rac4*. Sequencing was by the dideoxy chain termination method using a Perkin Elmer ABI377. The sequence analysis of pUC-*Rac4* revealed that the genomic clone contained a 2635 bp sequence 5' of the *Act2* translation start codon (*Rac2* = 1435 bp). In order to determine the structure of the *Act2* 5' region, a search was carried out of the Rice Genome Project's EST database with *Act2* 5' sequences. This sequence similarity search identified a partial cDNA sequence from callus tissue (D15626) that contains sequences identical

to two transcribed but untranslated exons in the 5' region of the *Act2* genomic clone. The search identified a partial cDNA sequence from rice callus tissue (D15626) that contains sequence identical to the two transcribed but untranslated exons, exon 1 and exon 2 in the 5' region of the *Act2* genomic clone. An alignment between the sequence of the *Act2* 5' region and the rice EST
 5 was used to determine the structure of the *Act2* 5' sequence. The 2635 bp sequence of the *Act2* 5' region was found to be composed of a promoter region of at least 740 bp, a 5' transcribed but untranslated first exon of at least 130 bp, a 5' intron of 1755 bp and the 14 bp transcribed but untranslated part of the second exon adjacent to the *Act2* protein's translation initiation site (FIG. 1, SEQ ID NO:1). The 5' intron contains a ~300 bp mini transposable element (MITE) of the
 10 *Tourist* (C) type. The *Act2* sequence in pUC-*RAc2* contains 1.45 kb of *Act2* 5' sequence and starts just upstream of the potential *Tourist* element within the *Act2* 5' intron.

The sequence around the translation start codon of the *Act2* gene was then modified to facilitate the construction of a translation fusion with the *gus* reporter gene (McElroy *et al.*, 1995), and the subsequent use of the *Act2* 5' region in transcriptional and translational fusion
 15 genes. The wild type, modified and maize consensus sequences are as given below, and in SEQ ID NO:4-SEQ ID NO:9.

Wild-type <i>Act2</i> 5' intron splice junctions and start codon region	
<u>CTGCAGCCGCCATCCCCGGTTCTCTCCTCTTCTTTAG</u> /gtgagcaa	
<i>Pst</i> I	
Modified <i>Act</i> 5' intron splice junction and start codon region:	
<u>CTGCAGCTGCCATCCCCGGTTCTCTCCTCTTCTTTAG</u> /gtaaccaa	
<i>Pst</i> I <i>Pvu</i> II	<i>Bst</i> EII
<i>Zea mays</i> consensus intron splice junction:	
AG/gtaagtnn	

Wild-type <i>Act2</i> 3' intron splice junctions and start codon region: tttgtgtatgcag/ATCAGTTAAATAAATGG
Modified <i>Act1</i> 3' intron splice junction and start codon region: ttttttttgcag/ <u>GTCGACTAGGTACCATGG</u> <div style="text-align: center;"><i>Sall</i> <i>KpnI</i> <i>NcoI</i></div>
<i>Zea mays</i> consensus 3' intron splice junction and start codon region: ttttttttgcag/GT ACAATGG

EXAMPLE 2

Preparation of Microprojectiles

5 Microprojectiles were prepared as follows: gold particles were prepared by adding 60 mg of 0.6 μm gold particles (BioRad, cat. no. 165-2262) to 1000 μl absolute ethanol and incubating for at least 3 hours at room temperature followed by storage at -20°C . Twenty to thirty five μl of the sterile gold particles and more preferably 30 to 35 μl of gold particles (30 μl contains 1.8 mg of particles) were centrifuged in a microcentrifuge for up to 1 min. The supernatant was removed and one ml sterile water was added to the tube, followed by centrifugation at 1800-2000 rpm for 2-5 minutes. Microprojectile particles were resuspended in 25-30 μl of DNA solution containing about 50 nM of DNA in a 30 μl solution.

15 Two hundred twenty microliters sterile water, 250 μl 2.5 M CaCl_2 and 50 μl stock spermidine (14 μl spermidine in 986 μl water) were then added to the particle containing solution. The solution was then thoroughly mixed and placed on ice, followed by vortexing at 4°C for 10 minutes and centrifugation at 500 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 600 μl absolute ethanol. Following centrifugation at 500 rpm for 5 minutes, the pellet was resuspended in 36-38 μl of absolute ethanol, vortexed for approximately 20 seconds, and sonicated for 20-30 seconds. At this stage the particles were typically allowed to sit for 2-5 minutes, after which 5-10 μl of the supernatant was removed and dispensed on the surface of a flyer disk and the ethanol was allowed to dry completely. Alternatively, particles may be removed directly after resuspension and vortexing 20 to 30 seconds in 36 μl -38 μl of ethanol, placed on the flyer disk and allowed to dry as done for the settled treatment. The

bombardment chamber was then evacuated to approximately 28 in. Hg prior to bombardment. The particles were then used for bombardment by a helium blast of approximately 1100 psi using the DuPont Biolistics PDS1000He particle bombardment device.

EXAMPLE 3

Bombardment of Hi-II Immature Embryos

Immature embryos (1.2 - 3.0 mm in length) of the corn genotype Hi-II were excised from surface-sterilized, greenhouse-grown ears of Hi-II 10-12 days post-pollination. The Hi-II genotype was developed from an A188 x B73 cross (Armstrong *et al.*, 1991). Approximately 30 embryos per petri dish were plated axis side down on a modified N6 medium containing 1 mg/l 2,4-D, 100 mg/l casein hydrolysate, 6 mM L-proline, 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES), 0.75 g/l MgCl₂, and 2% sucrose solidified with 2 g/l Gelgro, pH 5.8 (#735 medium). Embryos were cultured in the dark for two to four days at 24°C.

Approximately 3-4 hours prior to bombardment, embryos were transferred to the above culture medium with the sucrose concentration increased from 3% to 12%. When embryos were transferred to the high osmoticum medium they were arranged in concentric circles on the plate, starting 1 cm from the center of the dish, positioned such that their coleorhizal end was orientated toward the center of the dish. Usually two concentric circles were formed with 25-35 embryos per plate.

The plates containing embryos were placed on the third shelf from the bottom, 5 cm below the stopping screen. The 1100 psi rupture discs were used for bombardment. Each plate of embryos was bombarded once with the DuPont Biolistics PDS1000He particle gun. Following bombardment, embryos were allowed to recover on high osmoticum medium (735, 12% sucrose) overnight (16 - 24 hours) and were then transferred to selection medium containing 1 mg/l bialaphos (#739, 735 plus 1 mg/l bialaphos or #750, 735 plus 0.2M mannitol and 1 mg/l bialaphos). Embryos were maintained in the dark at 24° C. After three to four weeks on the initial selection plates about 90% of the embryos typically formed Type II callus and were transferred to selective medium containing 3 mg/l bialaphos (#758). Southern analysis can then be used for analysis of transformants and assays of gene expression may be carried out.

EXAMPLE 4**Transformation of H99 Immature Embryos or Callus
and Selection with Paromomycin**

Maize immature embryos (1.2 - 3.0 mm, 10-14 days post pollination) are isolated from
5 greenhouse grown H99 plants that have been self or sib pollinated. Immature embryos are
cultured on 735 medium in the dark at approximately 27°C. Immature embryos are either
bombarded 1-6 days after isolation or cultured to produce embryogenic callus that is used for
bombardment. Embryogenic callus is expanded and maintained by subculturing at 2-3 week
intervals to fresh 735 medium. Prior to bombardment, cultured embryos or embryogenic callus
10 (subdivided in approximately 2-4 mm clumps) are transferred to 735 medium containing 12%
sucrose for 3-6 hours. Following bombardment, carried out as described in Example 6, tissue
cultures are incubated overnight and transferred to 735 medium containing 500 mg/L
paromomycin. After 2-3 weeks, callus is subdivided into small pieces (approximately 2-4 mm in
diameter) and transferred to fresh selection medium. This subculture step is repeated at 2-3 week
15 intervals for up to about 15 week post-bombardment, with subdivision and visual selection for
healthy, growing callus.

Paromomycin tolerant callus is transferred to 735 medium without 2,4-D but containing
3.52 mg/L BAP for 3-9 days in the dark at approximately 27°C and is subsequently transferred to
110 medium (1/2X MS salts, 0.5 mg/L thiamine, 0.5 mg/L nicotinic acid, 3% sucrose, 3.6 g/L
20 Gelgro, pH 5.8) containing 100 mg/L paromomycin in Phytatrays (Sigma) and cultured at about
27°C in the light. Plantlets that develop in Phytatrays after 3-6 weeks are then transferred to soil.
Plantlets are acclimated in a growth chamber and grown to maturity in the greenhouse.

EXAMPLE 5**Expression Assays of *Act2* transformation Constructs**

25 A 4.3 kB *EcoRI-XbaI* restriction fragment, containing the *Act2* promoter, exon 1, intron
1 and exon 2, was isolated from *pUC-RAc4* and cloned in to pBSII-SK(-) (Stratagene) to create
the vector PBS-51RAc2. A 4.3 kB *SalI-SacII* restriction fragment, containing the *Act2*
promoter, exon 1, intron 1 and exon 2, was isolated from pBS-5'RAc2 and cloned into
pGEM5Zf(+) (Promega) to create the vector pGEM-5'RAc2. PCR-mediated sequence
30 mutagenesis was used to introduce *KpnI* and *NcoI* restriction sites around the gus translation

initiation codon, creating pGEM-PrAct2. A 2.7 kB *HinDIII-NcoI* restriction fragment, containing the *Act2* promoter, exon 1, intron 1 and part of exon 2, was isolated from pGEMPrAct2 and cloned into pSP-gus.n to create the vector pSP-Act2.gus.n. FIG. 4.

pSP-Act2.gus.n was digested with *BglII* (site at 1220 bp.) and *BclI* (site at 2059 bp.), the
5 intervening intron sequence containing the *Tourist* minitransposon-like inverted repeat excised, and the remaining sequence self-ligated to create pSP-Act2Δi.gus.n. FIG. 5.

A 4.5 kB *HpaI-EcoRI* restriction fragment, containing the *Act2* promoter (part), exon 1, intron 1, exon 2, along with gus and nos sequences, was isolated from pSP-Act2.gus.n and cloned into the *PvuII* and *EcoRI* sites of pSP72 (Promega) to create pSP-Act2.gus.n2. FIG. 6.

10 A 1.8 kB *PstI-NcoI* restriction fragment, containing the *Act2* exon 1 (part), intron 1 and exon 2, was isolated from pSP-Act2.gus.n and cloned into pS-gus.n to create pSP-Act2.gus.n3 (pSP-NGAct2). FIG. 7.

A 0.9 kB *PstI-NcoI* restriction fragment, containing the *Act2* exon 1 (part), intron 1 (deletion) and exon 2, was isolated from pSP-Act2Δi.gus.n and cloned into pSP-gus.n to create
15 the vector pSP-Act3.gus.n4 (pSP-NGAct2ΔI). FIG. 8.

A 3.6 kB *BglII-EcoRI* restriction fragment, containing the *Act2* promoter (part), exon 1, intron 1, exon 2, along with the gus and no sequences, was isolated from pSP-Act2.gus.n and cloned into the *BamHI* and *EcoRI* sites of pSP72 (Promega) to create pSP-Act2.gus.n5. FIG. 9.

A 4.5 kB *BclI-EcoRI* restriction fragment, containing the *Act2* promoter (part), exon 1,
20 intro 1, exon 2, along with gus and nos sequences, was isolated from pSP-Act2.gus.n and cloned into the *BamHI* and *EcoRI* sties of pSP72 (Promega) to create the vector pSP-Act2. gus.n6. FIG. 10.

The construction of pSP-gus.n (pDMC205) is described in McElroy *et al.* (1995). FIG.
11.

Transient assays were used to qualitatively determine the relative activity of the *Act2-gus* fusion gene when transformed into maize cells by microprojectile bombardment. First, microprojectiles were prepared as described in Example 2. The DNA used for transformation included an actin 1 promoter-gus fusion construct (McElroy *et al.*, 1995) and an actin 2-gus fusion construct (prepared as described in Example 1). Transformation of suspension cultures, callus root and leaf tissues was carried out using (1x6)716 cells (GII (A188x B73; SC716) as described in Example 3 and U.S. Patent 5,489,520.

The *Act2* 5' region showed constitutive activity in all cell types bombarded (Table 8), with a level of activity slightly lower than that of the 5' region of the rice actin 1 (*Act1*) gene (McElroy *et al.*, 1990) that is commonly used for high level constitutive transgene expression in transgenic cereal (Zhang *et al.*, 1991). *Act1-gus* was prepared as described in McElroy *et al.* (1991) (pAct-F4). *Act2-gus* is the same as pDPG834.

Table 8

Expression Analysis of Actin 1 and Actin 2 Regulatory Regions in Maize Tissues

Tissue	<i>Act1-gus</i>	<i>Act2-gus</i>
Suspension Culture	++++	++++
Callus	++++	not assayed
Root	+	+
Leaf	++	+

A study was next carried out to i) further quantify the activity of the *Act2* 5' region relative to that of the *Act1* 5' region; ii) identify the minimal amount of *Act2* promoter region necessary for full *Act2* 5' activity; iii) confirm the structure of the *Act2* 5' region; and iv) assay the effect of the putative *Tourist* element within the *Act2* intron on *Act2* 5' region activity. This study was carried out by transforming maize (1 × 6)716 cells with a series of actin 2 promoter

deletion mutants fused to the gus reporter gene. Transformation was carried out as described in Example 3.

Analysis of GUS activity by histochemical assays in transformed maize (1x6)716 cells indicated that: i) the *Act2* 5' region has ~75% the activity of the *Act1* 5' region; ii) the minimal amount of *Act2* 5' region necessary for full promoter activity is contained within a sequence 500 bp from the putative *Act2* transcription start site; iii) the *Act2* 5' region within the construct pUC-*RAct2* does not contain sufficient sequences for full *Act2* promoter activity; and iv) there is not significant effect on *Act2* 5' region activity associated with an internal deletion within the *Act2* intron which removed the putative *Tourist* element. Table 9.

Table 9

Histochemical Analysis of Gus Activity in Transformed Maize Cells

<u>Construct</u>	<u>GUS Specific Activity (nM 4-MU/μg protein/hr)</u>
pSP-Act2.gus.n	187.2 \pm 54.6
pSP-Act2 Δ I.gus.n	166.2 \pm 47.4
pSP-Act2.gus.n2	199.2 \pm 7.2
pSP-Act2.gus.n3	42.6 \pm 9.6
pSP-Act2.gus.n4	31.8 \pm 1.8
pSP-Act2.gus.n5	3.0 \pm 1.8
pSP-Act2.gus.n6	0.6 \pm 1.2
pSP-gus.n	0.0 \pm 0.6
Act1.gus.n	249.0 \pm 6.3

A study was next carried out to understand the expression profile of the actin 2 promoter in stably transformed plants. Transgenic plants were prepared by microprojectile bombardment of embryogenic maize tissues ((1x6)716 cells) with the *Act2-gus* gene (along with a *bar* selectable marker gene) to determine its pattern of activity in transgenic plants. Numerous transformants growing on bialaphos were recovered and a histochemical GUS assay was used to determine which transformants also contained a functional *Act2-gus* gene. Thirty three GUS

positive transformants were identified (Table 10, below). The *Act2-gus* reporter gene demonstrated high levels of GUS activity in the transformed maize callus.

Table 10
Gus Expression in Maize Callus

	# <i>bar</i> +	Transformation	# <i>gus</i> + Transformants
Study	Transformants	Efficiency	Identified
CS93PT	36/450	8%	14
IH60PT	48/238	20%	19

5

Analysis of bialaphos resistant maize callus derived from material co-transformed with the *Act2-gus* reporter gene confirmed a correlation between bialaphos resistance/GUS activity and the detection of *Bar/gus* PCRTM products. Maize callus transformed with the *Act2-gus* reporter gene demonstrated a high level of GUS activity in those parts of the callus that were undergoing embryogenesis.

10

A number of *Act2-gus* transgenic maize lines were selected for regeneration in order to determine the pattern of *Act2* 5' region (promoter, 5' exon and 5' intron) activity in transgenic maize plants. Regenerating plantlets derived from callus transformed with the *Act2-gus* reporter gene demonstrated high levels of GUS activity in the shoot apical meristem region and weaker GUS activity in the root and leaf vascular tissue. In order to evaluate the efficacy of the *Act2* promoter and 5' intron for gene expression in transgenic maize a number of plant lines containing an *Act2-gus-nos* reporter gene were produced from the regenerated transgenic plantlets. Putative "clonal" plants regenerated from 12 events described in Table 9 (*Act2-gus-nos*, in pDPG834 + 35S-*bar-nos*, in pDPG165; U.S. Patent No. 5,489,520) were transferred from the Shearer, into the greenhouse and onto pollination.

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Segregation of *Act2-gus-nos* reporter gene expression was evaluated in R₁ kernels from a

CS93PT1302 × DJ, a DEKALB elite inbred cross, 17 days after pollination. Of 19 kernels scored, 8 showed GUS activity in pericarp but not in embryo or endosperm, while 11 showed strong GUS activity in pericarp, embryo and endosperm. This ratio did not statistically differ from the expected 1:1 segregation ratio as determined by a χ^2 test ($p = 0.05$). Mendelian segregation of *Act2-gus* reporter gene expression in R_1 kernels was also found in an analysis of five other independent transformation events. Expression of the *Act2-gus-nos* reporter gene was found to be restricted to vascular tissue in roots, shoots and leaves of developing (3 - 21 day old) R_1 progeny plants from the CS93PT1302 × DJ cross. The qualitative level of *Act2-gus-nos* reporter gene expression appeared to be less than that described for an *Act1-gus-nos* reporter gene in transgenic cereals, but the results indicate the utility of the *Act2* regulatory regions for expression of transgenes in plants (Zhang *et al.*, 1991).

EXAMPLE 6

Methods for Microprojectile Bombardment

Many variations in techniques for microprojectile bombardment are well known in the art and therefore deemed useful with the current invention. Exemplary procedures for bombardment are discussed in, for example, U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993, specifically incorporated herein by reference in its entirety. Examples of target tissues which may be used with the current invention include immature embryos, Type I callus, Type II callus, Type III callus, suspension cultures and meristematic tissue (U.S. Patent No. 5,736,369). Some genotypes which are especially useful for maize transformation are specifically disclosed herein above, as well as in, for example, U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993. Preferred genotypes will be those which are readily transformable and which also may be regenerated to yield a fertile transgenic plant.

Any method for acceleration of microprojectiles may potentially be used to transform a plant cell with the current invention. A preferred method will be a gas-driven particle gun such as the DuPont Biolistics PDS1000He particle bombardment device. Exemplary particles for bombardment include those comprised of tungsten, gold, platinum, and the like. Gold particles are deemed particularly useful in the current invention, with 0.6 μm or 0.7 μm gold particles being preferred and 0.6 μm particles most preferred. The most preferred particles will be DNA coated and have a mean size between 0.6 μm and 1.0 μm . Alternatively, particles may be allowed

to settle for 2-5 minutes following precipitation of DNA onto particles. Particles are then removed from the supernatant and used for microprojectile bombardment. It is believed that the settling step enriches for a population of particles coated with DNA in which fewer aggregates of particles are present.

5

As disclosed herein, any DNA sequence may potentially be used for transformation. The DNA segments used for transformation will preferably include one or more selectable, secreteable or screenable markers. Many examples of such are well known in the art and are specifically disclosed herein. In the case of selectable markers, selection may be in solid or liquid media. The DNA segments used will preferably also include one or more genes which confer, either individually or in combination with other sequences, a desired phenotype on the transformed plant. Exemplary genes for transformation and the corresponding phenotypes these sequences may confer on the transformed plant are disclosed herein.

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EXAMPLE 7

Introgression of Transgenes Into Elite Inbreds and Hybrids

Backcrossing can be used to improve a starting plant. Backcrossing transfers a specific desirable trait from one source to an inbred or other plant that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question, for example, a construct prepared in accordance with the current invention. The progeny of this cross first are selected in the resultant progeny for the desired trait to be transferred from the non-recurrent parent, then the selected progeny are mated back to the superior recurrent parent (A). After five or more backcross generations with selection for the desired trait, the progeny are hemizygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other genes. The last backcross generation would be selfed to give progeny which are pure breeding for the gene(s) being transferred, *i.e.* one or more transformation events.

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Therefore, through a series a breeding manipulations, a selected transgene may be moved from one line into an entirely different line without the need for further recombinant manipulation. Transgenes are valuable in that they typically behave genetically as any other gene and can be

manipulated by breeding techniques in a manner identical to any other corn gene. Therefore, one may produce inbred plants which are true breeding for one or more transgenes. By crossing different inbred plants, one may produce a large number of different hybrids with different combinations of transgenes. In this way, plants may be produced which have the desirable agronomic properties frequently associated with hybrids ("hybrid vigor"), as well as the desirable characteristics imparted by one or more transgene(s).

EXAMPLE 8

Marker Assisted Selection

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

In the process of marker assisted breeding, DNA sequences are used to follow desirable agronomic traits (Tanksley *et al.*, 1989) in the process of plant breeding. Marker assisted breeding may be undertaken as follows. Seed of plants with the desired trait are planted in soil in the greenhouse or in the field. Leaf tissue is harvested from the plant for preparation of DNA at any point in growth at which approximately one gram of leaf tissue can be removed from the plant without compromising the viability of the plant. Genomic DNA is isolated using a procedure modified from Shure *et al.* (1983). Approximately one gram of leaf tissue from a seedling is lypholyzed overnight in 15 ml polypropylene tubes. Freeze-dried tissue is ground to a powder in the tube using a glass rod. Powdered tissue is mixed thoroughly with 3 ml extraction buffer (7.0 urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 3 ml phenol/chloroform. The aqueous phase is separated by centrifugation, and precipitated twice using 1/10 volume of 4.4 M ammonium

acetate pH 5.2, and an equal volume of isopropanol. The precipitate is washed with 75% ethanol and resuspended in 100-500 μ l TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

Genomic DNA is then digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20 SCP: 2M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). The filters are prehybridized in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 μ g/ml denatured salmon sperm DNA and 32 P-labeled probe generated by random priming (Feinberg & Vogelstein, 1983). Hybridized filters are washed in 2X SCP, 1% SDS at 65° for 30 minutes and visualized by autoradiography using Kodak XAR5 film. Genetic polymorphisms which are genetically linked to traits of interest are thereby used to predict the presence or absence of the traits of interest.

Those of skill in the art will recognize that there are many different ways to isolate DNA from plant tissues and that there are many different protocols for Southern hybridization that will produce identical results. Those of skill in the art will recognize that a Southern blot can be stripped of radioactive probe following autoradiography and re-probed with a different probe. In this manner one may identify each of the various transgenes that are present in the plant. Further, one of skill in the art will recognize that any type of genetic marker which is polymorphic at the region(s) of interest may be used for the purpose of identifying the relative presence or absence of a trait, and that such information may be used for marker assisted breeding.

Each lane of a Southern blot represents DNA isolated from one plant. Through the use of multiplicity of gene integration events as probes on the same genomic DNA blot, the integration event composition of each plant may be determined. Correlations may be established between the contributions of particular integration events to the phenotype of the plant. Only those plants that contain a desired combination of integration events may be advanced to maturity and used for pollination. DNA probes corresponding to particular transgene integration events are useful markers during the course of plant breeding to identify and combine particular integration events without having to grow the plants and assay the plants for agronomic performance.

It is expected that one or more restriction enzymes will be used to digest genomic DNA, either singly or in combinations. One of skill in the art will recognize that many different

restriction enzymes will be useful and the choice of restriction enzyme will depend on the DNA sequence of the transgene integration event that is used as a probe and the DNA sequences in the genome surrounding the transgene. For a probe, one will want to use DNA or RNA sequences which will hybridize to the DNA used for transformation. One will select a restriction enzyme that produces a DNA fragment following hybridization that is identifiable as the transgene integration event. Thus, particularly useful restriction enzymes will be those which reveal polymorphisms that are genetically linked to specific transgenes or traits of interest.

EXAMPLE 9

General Methods for Assays

DNA analysis of transformed plants is performed as follows. Genomic DNA is isolated using a procedure modified from Shure, *et al.*, 1983. Approximately 1 gm callus or leaf tissue is ground to a fine powder in liquid nitrogen using a mortar and pestle. Powdered tissue is mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 4 ml phenol/ chloroform. The aqueous phase is separated by centrifugation, passed through Miracloth, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate is washed with 70% ethanol and resuspended in 200-500 μ l TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

The presence of a DNA sequence in a transformed cell may be detected through the use of polymerase chain reaction (PCR). Using this technique specific fragments of DNA can be amplified and detected following agarose gel electrophoresis. For example, two hundred to 1000 ng genomic DNA is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 μ M each dATP, dCTP, dGTP, dTTP, 0.5 μ M each forward and reverse DNA primers, 20% glycerol, and 2.5 units Taq DNA polymerase. The reaction is run in a thermal cycling machine as follows: 3 minutes at 94 C, 39 repeats of the cycle 1 minute at 94 C, 1 minute at 50 C, 30 seconds at 72 C, followed by 5 minutes at 72 C. Twenty μ l of each reaction mix is run on a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours. Using this procedure, for example, one may detect the presence of the *bar* gene, using the forward primer CATCGAGACAAGCACGGTCAACTTC (SEQ ID NO:10) and the reverse primer AAGTCCCTGGAGGCACAGGGCTTCAAGA (SEQ ID

NO:11). Primers for the *Act2* intron and promoter can be readily prepared by one of skill in the art in light of the *Act2* sequences given in SEQ ID NO:1.

A method to detect the presence of phosphinothricin acetyl transferase (PAT) involves the use of an *in vitro* enzyme reaction followed by thin layer chromatography, as described in U.S. Pat. Appl. No. 08/113,561, filed August 25, 1993 (specifically incorporated herein by reference in its entirety). The procedure is conducted by preparing various protein extracts from homogenates of potentially transformed cells, and from control cells that have neither been transformed nor exposed to bialaphos selection, and then assaying by incubation with PPT and ¹⁴C-Acetyl Coenzyme A followed by thin layer chromatography. The results of this assay provide confirmation of the expression of the *bar* gene which codes for PAT.

For Southern blot analysis genomic DNA is digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Probes are labeled with ³²P using the random priming method (Boehringer Mannheim) and purified using Quik-Sep® spin columns (Isolab Inc., Akron, OH). Filters are prehybridized at 65°C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml heparin (Chomet *et al.*, 1987) for 15 min. Filters then are hybridized overnight at 65 C in 6X SCP containing 100 µg/ml denatured salmon sperm DNA and ³²P-labeled probe. Filters are washed in 2X SCP, 1% SDS at 65 C for 30 min. and visualized by autoradiography using Kodak XAR5 film. For rehybridization, the filters are boiled for 10 min. in distilled H₂O to remove the first probe and then prehybridized as described above.

EXAMPLE 10

Utilization of Transgenic Crops

The ultimate goal in plant transformation is to produce plants which are useful to man. In this respect, transgenic plants created in accordance with the current invention may be used for virtually any purpose deemed of value to the grower or to the consumer. For example, one may wish to harvest seed from transgenic plants. This seed may in turn be used for a wide variety of purposes. The seed may be sold to farmers for planting in the field or may be directly used as food, either for animals or humans. Alternatively, products may be made from the seed itself.

Examples of products which may be made from the seed include, oil, starch, animal or human food, pharmaceuticals, and various industrial products. The food uses of maize, in addition to human consumption of maize kernels, include both products of dry- and wet-milling industries. The principal products of maize dry milling are grits, meal and flour. The maize wet-milling industry can provide maize starch, maize syrups, and dextrose for food use. Maize oil is recovered from maize germ, which is a by-product of both dry- and wet-milling industries.

Maize, including both grain and non-grain portions of the plant, also is used extensively as livestock feed, primarily for beef cattle, dairy cattle, hogs, and poultry. Industrial uses of maize include production of ethanol, maize starch in the wet-milling industry and maize flour in the dry-milling industry. The industrial applications of maize starch and flour are based on functional properties, such as viscosity, film formation, adhesive properties, and ability to suspend particles. The maize starch and flour have application in the paper and textile industries. Other industrial uses include applications in adhesives, building materials, foundry binders, laundry starches, explosives, oil-well muds, and other mining applications. Plant parts other than the grain of maize also are used in industry, for example, stalks and husks are made into paper and wallboard and cobs are used for fuel and to make charcoal. Other means for utilizing plants, such as those that may be made with the current invention, have been well known since the dawn of agriculture and will be known to those of skill in the art in light of the instant disclosure. Specific methods for crop utilization may be found in, for example, Sprague and Dudley (1988), and Watson and Ramstad (1987).

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All of the compositions and methods disclosed and claimed herein can be made and
executed without undue experimentation in light of the present disclosure. While the
25 compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied to the
compositions and methods and in the steps or in the sequence of steps of the methods described
herein without departing from the concept, spirit and scope of the invention. More specifically, it
will be apparent that certain agents which are both chemically and physiologically related may be
30 substituted for the agents described herein while the same or similar results would be achieved.
All such similar substitutes and modifications apparent to those skilled in the art are deemed to be
within the spirit, scope and concept of the invention as defined by the appended claims.

CLAIMS:

1. An isolated rice actin 2 promoter isolatable from the nucleic acid sequence of SEQ ID NO:1.
- 5 2. An isolated rice actin 2 promoter isolatable from the nucleic acid sequence of SEQ ID NO:2.
3. An isolated nucleic acid comprising from about 40 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
- 10 4. The isolated nucleic acid of claim 3, comprising from about 60 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
- 15 5. The isolated nucleic acid of claim 4, comprising from about 125 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
6. The isolated nucleic acid of claim 5, comprising from about 250 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
- 20 7. The isolated nucleic acid of claim 6, comprising from about 400 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
8. The isolated nucleic acid of claim 7, comprising from about 600 to about 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.
- 25 9. The isolated nucleic acid of claim 8, comprising the nucleic acid sequence of SEQ ID NO:2.
- 30 10. An isolated rice actin 2 intron isolatable from the nucleic acid sequence of SEQ ID NO:1.
11. An isolated rice actin 2 intron isolatable from the nucleic acid sequence of SEQ ID NO:3.

12. An isolated nucleic acid comprising from about 40 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

5 13. The isolated nucleic acid of claim 12, comprising from about 100 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

14. The isolated nucleic acid of claim 13, comprising from about 300 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

10

15. The isolated nucleic acid of claim 14, comprising from about 700 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

16. The isolated nucleic acid of claim 15, comprising from about 1200 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

15

17. The isolated nucleic acid of claim 16, comprising from about 1500 to about 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

20 18. The isolated nucleic acid of claim 17, comprising the nucleic acid sequence of SEQ ID NO:3.

19. An expression vector comprising an isolated rice actin 2 promoter.

25 20. The expression vector of claim 19, wherein said rice actin 2 promoter is isolatable from the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

21. The expression vector of claim 19, wherein said rice actin 2 promoter is operably linked to a selected gene.

30

22. The expression vector of claim 21, wherein said selected gene is an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance

gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

5

23. The expression vector of claim 22, wherein said selected gene is a selectable marker gene encoding a protein selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant
10 nitrilase and anthranilate synthase.

24. The expression vector of claim 22, wherein said selected gene is operably linked to a terminator.

15 25. The expression vector of claim 21, further comprising a genetic element which enhances the expression of said selected gene when said expression vector is stably transformed in the genome of a host plant.

26. The expression vector of claim 25, wherein said genetic element is selected from the
20 group consisting of the rice actin 1 intron and the rice actin 2 intron.

27. The expression vector of claim 26, wherein said genetic element is the rice actin 2 intron.

28. The expression vector of claim 19, wherein said expression vector comprises a plasmid.
25

29. The expression vector of claim 28, wherein said rice actin 2 promoter is located on an expression cassette isolated from said plasmid.

30. The expression vector of claim 19, wherein said vector comprises a transit peptide.
30

31. The expression vector of claim 30, wherein said transit peptide is selected from the group consisting of chlorophyll a/b binding protein transit peptide, small subunit of ribulose

bisphosphate carboxylase transit peptide, EPSPS transit peptide and dihydrodipicolinic acid synthase transit peptide.

32. An expression vector comprising an isolated rice actin 2 intron.

33. The expression vector of claim 32, wherein said rice actin 2 intron is isolatable from the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3.

34. The expression vector of claim 32, further comprising a promoter operable in plants.

35. The expression vector of claim 34, wherein said promoter operable in plants is a promoter selected from the group consisting of a gamma zein promoter, an oleosin ole16 promoter, a globulin1 promoter, an actin 1 promoter, an actin c1 promoter, a sucrose synthetase promoter, an INOPS promoter, an EMB5 promoter, a globulin2 promoter, a b-32, ADPG-pyrophosphorylase promoter, an Ltp1 promoter, an Ltp2 promoter, an oleosin ole17 promoter, an oleosin ole18 promoter, an actin 2 promoter, a pollen-specific protein promoter, a pollen-specific pectate lyase promoter, an anther-specific protein promoter, an anther-specific gene RTS2 promoter, a pollen-specific gene promoter, a tapetum-specific gene promoter, tapetum-specific gene RAB24 promoter, a anthranilate synthase alpha subunit promoter, an alpha zein promoter, an anthranilate synthase beta subunit promoter, a dihydrodipicolinate synthase promoter, a Thi1 promoter, an alcohol dehydrogenase promoter, a cab binding protein promoter, an H3C4 promoter, a RUBISCO SS starch branching enzyme promoter, an ACCase promoter, an actin3 promoter, an actin7 promoter, a regulatory protein GF'14-12 promoter, a ribosomal protein L9 promoter, a cellulose biosynthetic enzyme promoter, an S-adenosyl-L-homocysteine hydrolase promoter, a superoxide dismutase promoter, a C-kinase receptor promoter, a phosphoglycerate mutase promoter, a root-specific RCc3 mRNA promoter, a glucose-6 phosphate isomerase promoter, a pyrophosphate-fructose 6-phosphate1phosphotransferase promoter, an ubiquitin promoter, a beta-ketoacyl-ACP synthase promoter, a 33kDa photosystem II promoter, an oxygen evolving protein promoter, a 69 kDa vacuolar ATPase subunit promoter, a metallothionein-like protein promoter, a glyceraldehyde-3-phosphate dehydrogenase promoter, an ABA- and ripening-inducible-like protein promoter, a phenylalanine ammonia lyase promoter, an adenosine triphosphatase S-adenosyl-L-homocysteine hydrolase promoter, an α -tubulin promoter, a cab

promoter, a PEPCase promoter, an R gene promoter, a lectin promoter, a light harvesting complex promoter, a heat shock protein promoter, a chalcone synthase promoter, a zein promoter, a globulin-1 promoter, an ABA promoter, an auxin-binding protein promoter, a UDP glucose flavonoid glycosyl-transferase gene promoter, an MPI promoter, an actin promoter, an opaque 2 promoter, a b70 promoter, an oleosin promoter, a CaMV 35S promoter, a CaMV 19S promoter, a histone promoter, a turgor-inducible promoter, a pea small subunit RuBP carboxylase promoter, a Ti plasmid mannopine synthase promoter, Ti plasmid nopaline synthase promoter, a petunia chalcone isomerase promoter, a bean glycine rich protein 1 promoter, a CaMV 35S transcript promoter, a Potato patatin promoter, and a S-E9 small subunit RuBP carboxylase promoter.

36. The expression vector of claim 32, further comprising an exogenous gene selected from the group consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

37. The expression vector of claim 36, wherein said selected gene is a selectable marker gene encoding a protein selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase and anthranilate synthase.

38. The expression vector of claim 37, wherein said selected gene is operably linked to a terminator.

39. The expression vector of claim 32, wherein said expression vector comprises a plasmid.

40. The expression vector of claim 39, wherein said rice actin 2 intron is located on an expression cassette isolated from said plasmid.

41. A fertile transgenic plant stably transformed with a selected DNA comprising an actin 2 promoter.

5 42. The fertile transgenic plant of claim 41, wherein said rice actin 2 promoter is isolatable from the nucleic acid sequence of SEQ ID NO:2.

43. The fertile transgenic plant of claim 41, wherein said selected DNA further comprises a selected gene operably linked to said rice actin 2 promoter.

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44. The fertile transgenic plant of claim 43, wherein said selected gene is an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

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45. The fertile transgenic plant of claim 44, wherein said selected gene is a selectable marker gene encoding a protein selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase and anthranilate synthase.

20

46. The fertile transgenic plant of claim 45, wherein said selected gene is operably linked to a terminator.

25

47. The fertile transgenic plant of claim 43, wherein said selected DNA further comprises a genetic element which enhances the expression of said selected gene in said fertile transgenic plant.

30

48. The fertile transgenic plant of claim 47, wherein said genetic element is selected from the group consisting of rice actin 1 intron and rice actin 2 intron.

49. The fertile transgenic plant of claim 48, wherein said genetic element is said rice actin 2 intron.

5 50. The fertile transgenic plant of claim 41, wherein said selected DNA comprises plasmid DNA.

51. The fertile transgenic plant of claim 50, wherein said selected DNA comprises an expression cassette isolated from said plasmid.

10 52. The fertile transgenic plant of claim 41, wherein said selected DNA comprises a transit peptide.

53. The fertile transgenic plant of claim 52, wherein said transit peptide is selected from the
15 group consisting of chlorophyll a/b binding protein transit peptide, small subunit of ribulose biphosphate carboxylase transit peptide, EPSPS transit peptide and dihydrodipicolinic acid synthase transit peptide.

54. The fertile transgenic plant of claim 41, wherein said plant is a monocotyledonous plant.

20 55. The fertile transgenic plant of claim 54, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

56. The fertile transgenic plant of claim 41, wherein said plant is a dicotyledonous plant.

25 57. The fertile transgenic plant of claim 56, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

58. A crossed fertile transgenic plant prepared according to the method comprising the steps
30 of:

- (i) obtaining a fertile transgenic plant comprising a selected DNA comprising an actin 2 promoter;

- (ii) crossing said fertile transgenic plant with itself or with a second plant lacking said selected DNA to prepare the seed of a crossed fertile transgenic plant comprising said selected DNA; and
- (iii) planting said seed to obtain a crossed fertile transgenic plant.

5

59. The crossed fertile transgenic plant of claim 58, wherein said crossed fertile transgenic plant is a monocotyledonous plant.

60. The crossed fertile transgenic plant of claim 59, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

10

61. The crossed fertile transgenic plant of claim 60, wherein said monocotyledonous plant is a maize plant.

15

62. The crossed fertile transgenic plant of claim 58, wherein said crossed fertile transgenic plant is a dicotyledonous plant.

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63. The crossed fertile transgenic plant of claim 62, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, sunflower, alfalfa, canola and cotton.

25

64. The crossed fertile transgenic plant of claim 58, wherein said selected DNA is inherited through a female parent.

65. The crossed fertile transgenic plant of claim 58, wherein said selected DNA is inherited through a male parent.

30

66. The crossed fertile transgenic plant of claim 58, wherein said second plant is an inbred plant.

67. The crossed fertile transgenic plant of claim 66, wherein said crossed fertile transgenic plant is a hybrid.

5 68. The crossed fertile transgenic plant of claim 58, wherein said actin 2 promoter is isolatable from the nucleic acid sequence of SEQ ID NO:2

69 The crossed fertile transgenic plant of claim 58, wherein said actin 2 promoter comprises from about 40 to 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

10 70. The crossed fertile transgenic plant of claim 69, wherein said actin 2 promoter comprises from about 100 to 743 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

71. The crossed fertile transgenic plant of claim 58, wherein said selected DNA further comprises an exogenous gene operably linked to said actin 2 promoter.

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72. The crossed fertile transgenic plant of claim 71, wherein said exogenous gene is selected from the group consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization
20 gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

73. Seeds of the crossed fertile transgenic plant of claim 58.

25

74. A crossed fertile transgenic plant prepared according to the method comprising:

- (i) obtaining a fertile transgenic plant comprising a selected DNA comprising an actin 2 intron;
- (ii) crossing said fertile transgenic plant with itself or with a second plant lacking said selected DNA to prepare seed of a crossed fertile transgenic plant comprising said selected DNA; and
- (iii) planting said seed to obtain a crossed fertile transgenic plant comprising said selected DNA.

75. The crossed fertile transgenic plant of claim 74, wherein said crossed fertile transgenic plant is a monocotyledonous plant.

76. The crossed fertile transgenic plant of claim 75, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

77. The crossed fertile transgenic plant of claim 76, wherein said monocotyledonous plant is a maize plant.

78. The crossed fertile transgenic plant of claim 74, wherein said crossed fertile transgenic plant is a dicotyledonous plant.

79. The crossed fertile transgenic plant of claim 78, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, sunflower, alfalfa, canola and cotton.

80. The crossed fertile transgenic plant of claim 74, wherein said selected DNA is inherited through a female parent.

81. The crossed fertile transgenic plant of claim 74, wherein said selected DNA is inherited through a male parent.

82. The crossed fertile transgenic plant of claim 74, wherein said second plant is an inbred plant.

83. The crossed fertile transgenic plant of claim 82, wherein said crossed fertile transgenic
5 plant is a hybrid.

84. The crossed fertile transgenic plant of claim 74, wherein said actin 2 intron is isolatable from the nucleic acid sequence of SEQ ID NO:3.

10 85. The crossed fertile transgenic plant of claim 74, wherein said actin 2 intron comprises from about 40 to 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

86. The crossed fertile transgenic plant of claim 85, wherein said actin 2 intron comprises from about 100 to 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

15

87. The crossed fertile transgenic plant of claim 86, wherein said actin 2 intron comprises from about 200 to 1763 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:3.

88. The crossed fertile transgenic plant of claim 74, wherein said selected DNA further
20 comprises an exogenous gene.

89. The crossed fertile transgenic plant of claim 88, wherein said exogenous gene is selected from the group consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a herbicide resistance gene, a gene
25 affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

30 90. Seeds of the crossed fertile transgenic plant of claim 74.

91. A method of expressing an exogenous gene in a plant comprising the steps of:

- (i) preparing a construct comprising said exogenous gene operably linked to an actin 2 promoter;
- (ii) transforming a recipient plant cell with said construct; and
- 5 (iii) regenerating a transgenic plant expressing said exogenous gene from said recipient cell.

92. The method of claim 91, wherein said recipient plant cell is from a monocotyledonous plant.

93. The method of claim 92, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

94. The method of claim 93, wherein the monocotyledonous plant is a maize plant.

95. The method of claim 91, wherein said recipient plant cell is from a dicotyledonous plant.

96. The method of claim 95, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, sunflower, canola, alfalfa and cotton.

97. The method of claim 96, wherein said exogenous gene is selected from the group consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

98. A method of expressing an exogenous gene in a plant comprising the steps of:

- (i) preparing a construct comprising an actin 2 intron and an exogenous gene;
- (ii) transforming a recipient plant cell with said construct; and

- (iii) regenerating a transgenic plant expressing said exogenous gene from said recipient cell.

99. The method of claim 98, wherein said recipient plant cell is from a monocotyledonous plant.

100. The method of claim 99, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

101. The method of claim 100, wherein the monocotyledonous plant is a maize plant.

102. The method of claim 98, wherein said recipient plant cell is from a dicotyledonous plant.

103. The method of claim 102, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, sunflower, alfalfa, canola and cotton.

104. The method of claim 98, wherein said construct comprises a promoter operably linked to said exogenous gene.

105. The method of claim 104, wherein said promoter is selected from the group consisting of a gamma zein promoter, an oleosin ole16 promoter, a globulin1 promoter, an actin 1 promoter, an actin c1 promoter, a sucrose synthetase promoter, an INOPS promoter, an EMB5 promoter, a globulin2 promoter, a b-32, ADPG-pyrophosphorylase promoter, an Ltp1 promoter, an Ltp2 promoter, an oleosin ole17 promoter, an oleosin ole18 promoter, an actin 2 promoter, a pollen-specific protein promoter, a pollen-specific pectate lyase promoter, an anther-specific protein promoter, an anther-specific gene RTS2 promoter, a pollen-specific gene promoter, a tapetum-specific gene promoter, tapetum-specific gene RAB24 promoter, a anthranilate synthase alpha subunit promoter, an alpha zein promoter, an anthranilate synthase beta subunit promoter, a dihydrodipicolinate synthase promoter, a Th1l promoter, an alcohol dehydrogenase promoter, a cab binding protein promoter, an H3C4 promoter, a RUBISCO SS starch branching enzyme promoter, an ACCase promoter, an actin3 promoter, an actin7 promoter, a regulatory protein GF 14-12 promoter, a ribosomal protein L9 promoter, a cellulose biosynthetic enzyme promoter,

an S-adenosyl-L-homocysteine hydrolase promoter, a superoxide dismutase promoter, a C-kinase receptor promoter, a phosphoglycerate mutase promoter, a root-specific RCc3 mRNA promoter, a glucose-6 phosphate isomerase promoter, a pyrophosphate-fructose 6-phosphate phosphotransferase promoter, an ubiquitin promoter, a beta-ketoacyl-ACP synthase promoter, a 33kDa photosystem II promoter, an oxygen evolving protein promoter, a 69 kDa vacuolar ATPase subunit promoter, a metallothionein-like protein promoter, a glyceraldehyde-3-phosphate dehydrogenase promoter, an ABA- and ripening-inducible-like protein promoter, a phenylalanine ammonia lyase promoter, an adenosine triphosphatase S-adenosyl-L-homocysteine hydrolase promoter, an α -tubulin promoter, a cab promoter, a PEPCase promoter, an R gene promoter, a lectin promoter, a light harvesting complex promoter, a heat shock protein promoter, a chalcone synthase promoter, a zein promoter, a globulin-1 promoter, an ABA promoter, an auxin-binding protein promoter, a UDP glucose flavonoid glycosyl-transferase gene promoter, an MPI promoter, an actin promoter, an opaque 2 promoter, a b70 promoter, an oleosin promoter, a CaMV 35S promoter, a CaMV 19S promoter, a histone promoter, a turgor-inducible promoter, a pea small subunit RuBP carboxylase promoter, a Ti plasmid mannopine synthase promoter, Ti plasmid nopaline synthase promoter, a petunia chalcone isomerase promoter, a bean glycine rich protein 1 promoter, a CaMV 35S transcript promoter, a Potato patatin promoter, and a S-E9 small subunit RuBP carboxylase promoter.

106. The method of claim 98, wherein said exogenous gene is selected from the group consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

107. A method of plant breeding comprising the steps of:

- (i) obtaining a transgenic plant comprising a selected DNA comprising an actin 2 promoter; and
- (ii) crossing said transgenic plant with itself or a second plant.

108. The method of claim 107, wherein said second plant is an inbred plant.

109. The method of claim 107, wherein said transgenic plant is a monocotyledonous plant.

5 110. The method of claim 109, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

111. The method of claim 110, wherein the monocotyledonous plant is a maize plant.

10 112. The method of claim 107, wherein said transgenic plant is a dicotyledonous plant.

113. The method of claim 112, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

15 114. A method of plant breeding comprising the steps of:

- (i) obtaining a transgenic plant comprising a selected DNA comprising an actin 2 intron; and
- (ii) crossing said transgenic plant with itself or a second plant.

20 115. The method of claim 114, wherein said second plant is an inbred plant.

116. The method of claim 114, wherein said transgenic plant is a monocotyledonous plant.

117. The method of claim 116, wherein said monocotyledonous plant is selected from the
25 group consisting of wheat, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

118. The method of claim 117, wherein the monocotyledonous plant is a maize plant.

119. The method of claim 114, wherein said transgenic plant is a dicotyledonous plant.

30

120. The method of claim 119, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

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FIG. 1

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FIG. 1, cont'd.

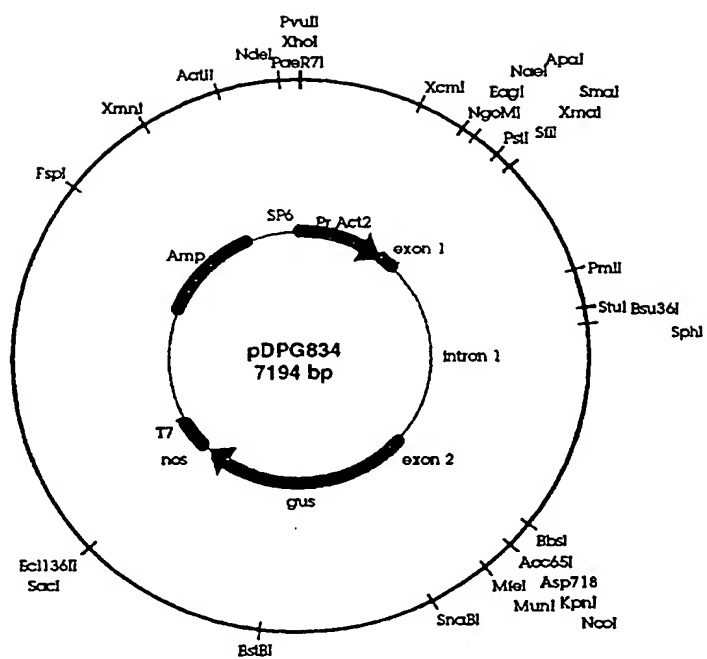


FIG. 2

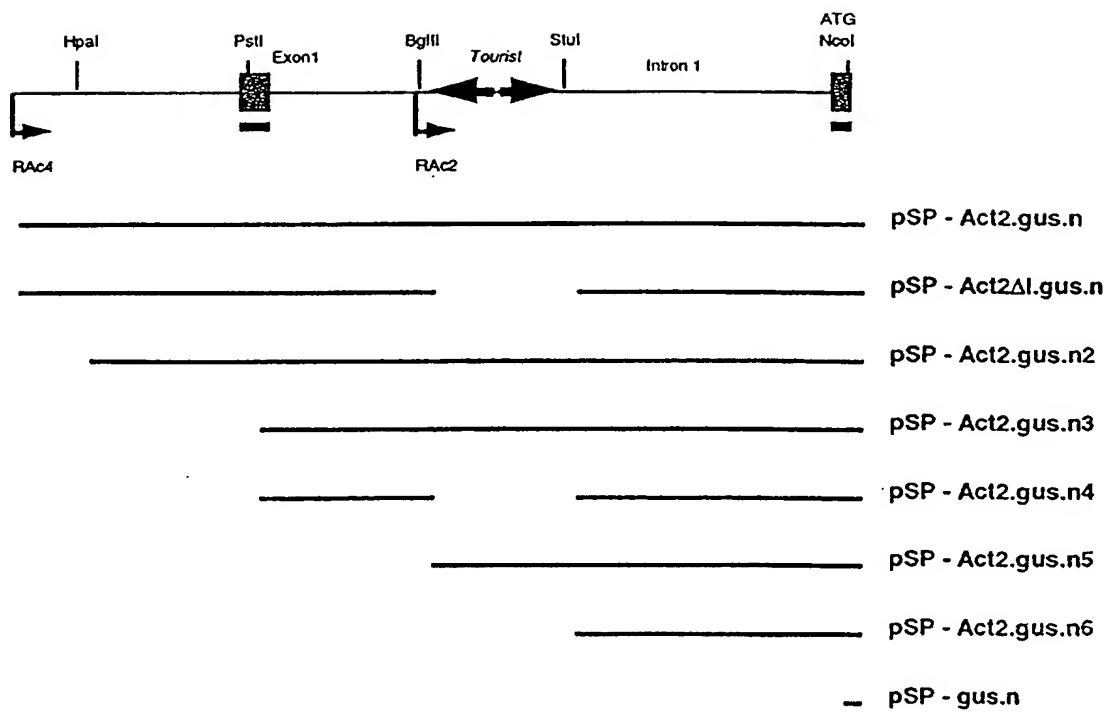


FIG. 3



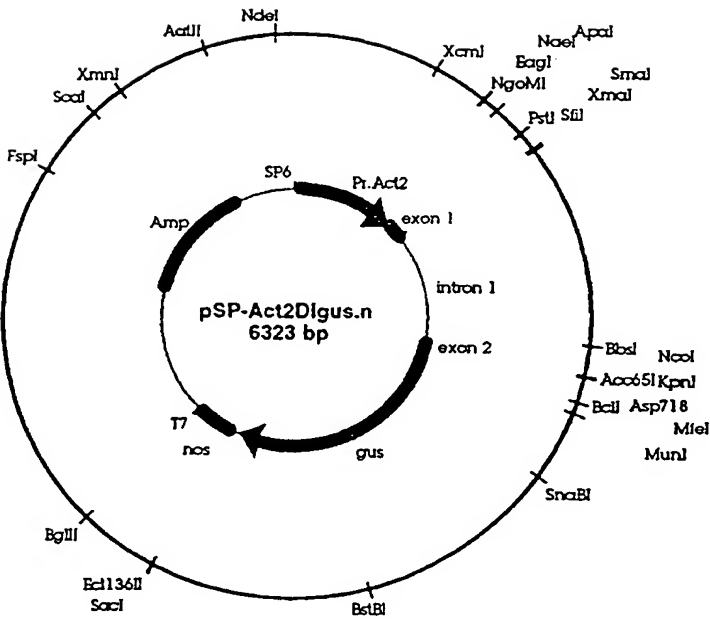


FIG. 5

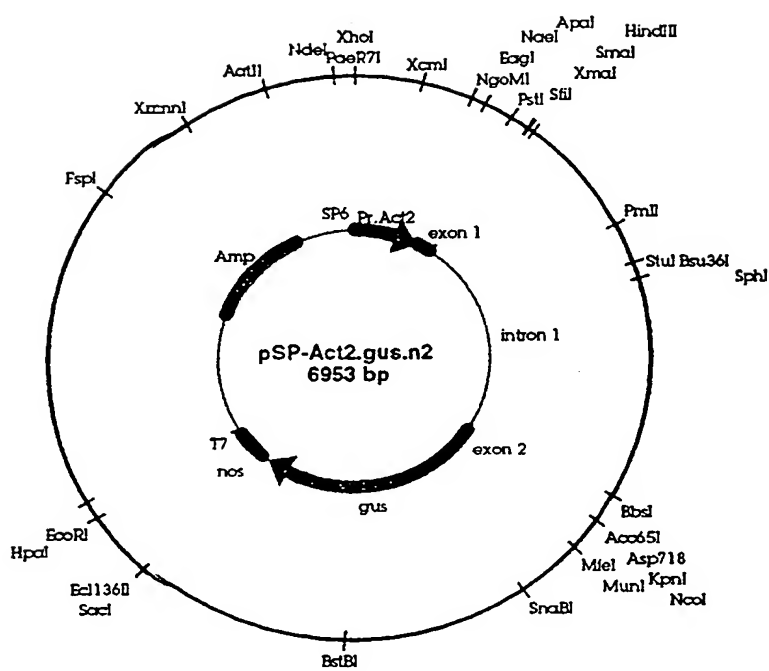


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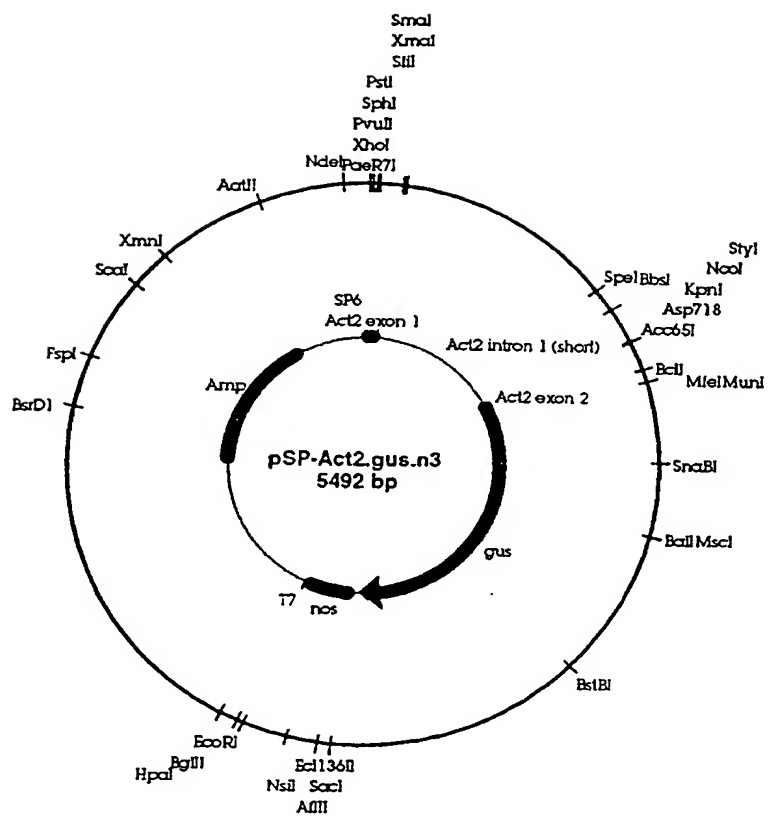


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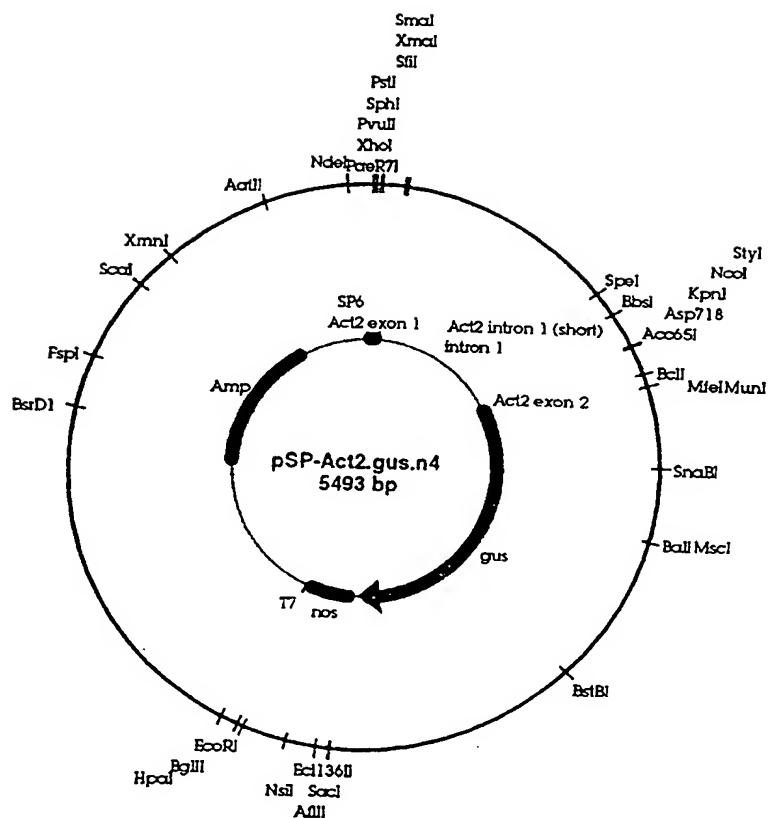


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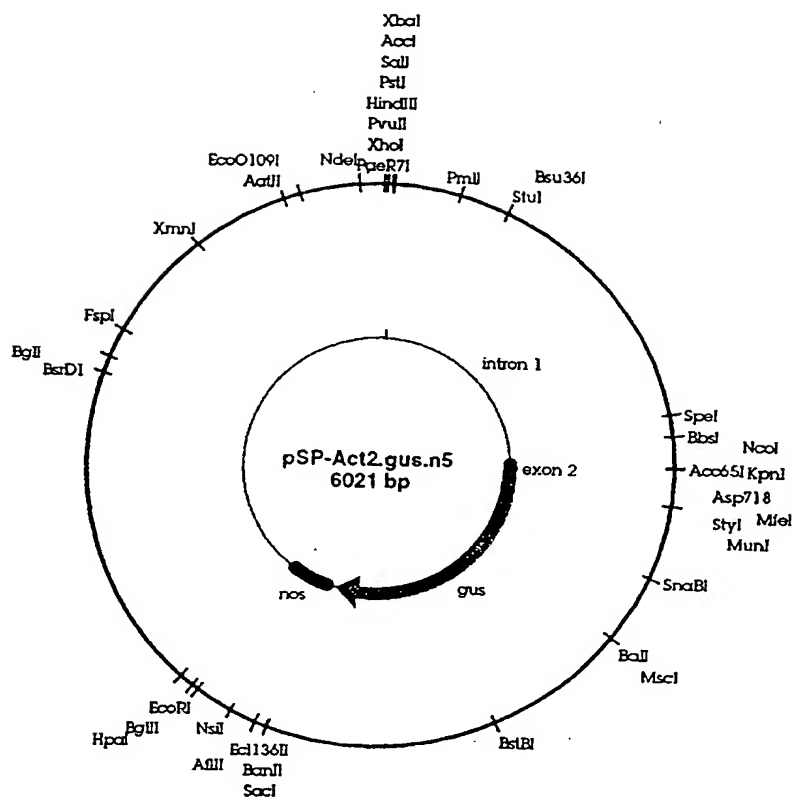


FIG. 9

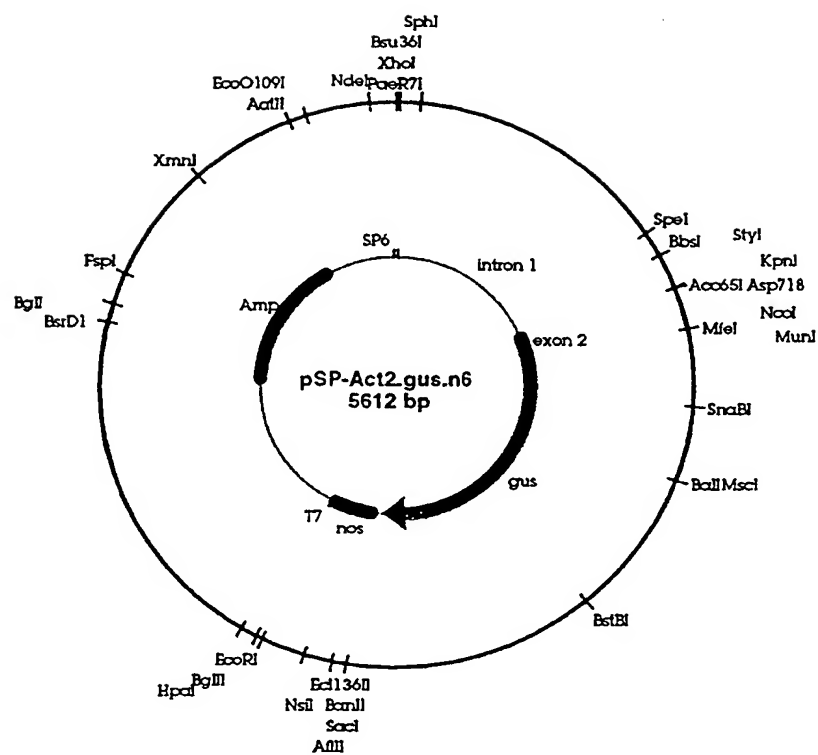


FIG. 10

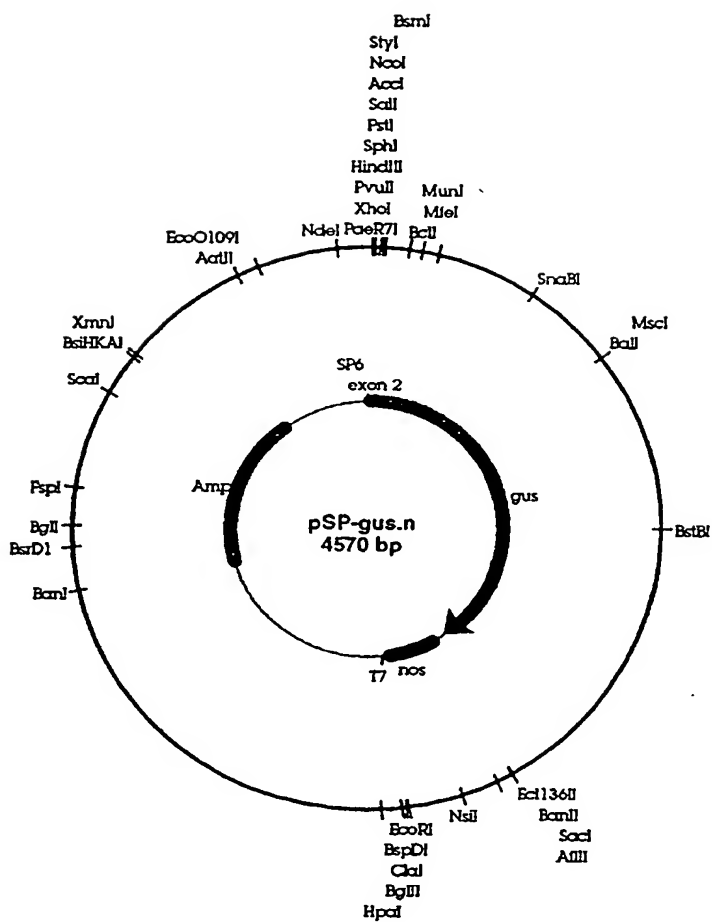


FIG. 11

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WU, RAY

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25 <400> 4
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20 <220>
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<223> Note: N = A, C, G, or T

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